KININS AND KININ RECEPTORS IN THE PATHOGENESIS OF HEART FAILURE

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Doctoral thesis
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Helsinki, Finland

Academic Dissertation

To be presented by permission of the Medical faculty of the University of Helsinki, for public examination in auditorium 4 at Meilahti Hospital on December 5th 2002 at 12 noon.

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ISBN 952-91-5388-0 (paperback)

ALSO AVAILABLE IN ELECTRONIC FORMAT
ISBN 952-10-0834-2 (PDF)
URL:http://ethesis.helsinki.fi/

Yliopistopaino
Helsinki 2002

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1. ABBREVIATIONS AND ACRONYMS

3H-BK tritium-labeled bradykinin
ACE angiotensin-converting enzyme
ACEi ACE inhibitor
Ang II angiotensin II
AP-1 activator protein 1
APM aminopeptidase M
APP aminopeptidase P
AT_1 angiotensin II type 1 receptors
AT_2 angiotensin II type 2 receptors
BCG mycobacterium bovis bacillus Calmette-Guerin vaccination
BK bradykinin
BK-1R bradykinin type 1 receptors
BK-2R bradykinin type 2 receptors
BK-2R-KO BK-2R knockout mouse
BSA bovine serum albumin
cAMP cyclic adenosin monophosphate
cGMP cyclic guanosin monophosphate
CHD coronary heart disease
CHF congestive heart failure
CK creatinine kinase
CMC cardiomyocytes
COX2 cyclo-oxygenase, type 2
CPM carboxypeptidase M
CPN carboxypeptidase N
CREB cAMP responsive element binding protein
DOCA deoxycortisone-acetate (salt)
E/A early rapid diastolic filling wave/late diastolic filling wave
EC endothelial cells
EDHF endothelium-derived hyperpolarizing factor
EET epoxyeicosatrienoic acid
EGF epidermal growth factor
eNOS endothelial nitric oxide synthase
eNOS-KO eNOS-knockout mouse
ERK/ELK extracellular-signal regulated kinase
FAP furanacryloyl-Phe
FAPGG furanacryloyl-Phe-Gly-Gly
GAPDH glyceraldehyde-3-phosphate dehydrogenase
GLUT4 glucose transporter protein 4
HF heart failure
HMW kininogen high-molecular-weight kininogen
HOE-140 BK-2R antagonist
IDC idiopathic dilated cardiomyopathy
IFN-γ interferon γ
IKK2 IκB kinase 2
IL-1β interleukin 1β
IL-6 Interleukin type 6
IL-8 Interleukin type 8
iNOS inducible nitric oxide synthase
IP_3 inositol triphosphate
IVS interventricular septum
JAK Janus kinase
KD kallidin
KKK kininogen-kallikrein-kinin (system)
KLK kallikrein
LMW kininogen low-molecular-weight kininogen
LPS lipopolysaccharide

LVESD left ventricular end-systolic diameter
LVEDD left ventricular end-diastolic diameter
LVFS left ventricular shortening fraction
LVH left ventricular hypertrophy
MAP kinase mitogen-activated protein (kinase)
MEK MAP kinase kinase
MGEA DL-2-Mercaptomethyl-3-guanidino ethylthiopropanoic acid
MI myocardial infarction
mRNA messenger ribonucleic acid
NEP neutral endopeptidase
NEPi neutral endopeptidase inhibitor
NF-κB nuclear factor-κB
NO nitric oxide
NPR-A natriuretic peptide receptor A
Oct-1 octamer binding transcription factor I
PBS phosphate buffered saline
PC preconditioning
PDGF platelet-derived growth factor
PEA3 subfamily of Ets transcription factors
PGE_2 prostaglandin E2
PGI_2 prostacyclin
PI3K phosphoinositide 3 kinase
Pif-1a PMA-inducible factor Ia
PKC protein kinase C
pKLK plasma kallikrein or prekallikrein, kallikrein B1
PLA_2 phospholipase A2
PLC phospholipase C
PLD phospholipase D
PW (thickness) posterior wall
Raf-1 proto-oncogene serine/threonine-protein kinase (MAP kinase kinase kinase)
RAS renin-angiotensin-aldosterone (system)
RIA radioimmunoassay
ROS reactive oxygen species
RP-HPLC reverse-phase high-performance liquid chromatography
RT-PCR reverse transcriptase-polymerase chain reaction
SAPK stress-activated protein kinase
SD rat Sprague-Dawley rat
SDS-PAGE sodium-dodecyl-sulfate-polyacryl-amide gel
SHR spontaneously hypertensive rat
SMC smooth muscle cell
SP I specificity protein I
STAT signal transducer and activator of transcription
STZ-rats rats with streptozotocin-induced diabetes
TGF-β transforming growth factor β
TIMP tissue inhibitor of matrix metalloproteinases
tKLK tissue kallikrein, kallikrein 1, true kallikrein
TNF-α tumor necrosis factor α
tyk2 Janus kinase type tyrosine kinase of the JAK/STAT pathway
VHD valvular heart disease
WKY Wistar Kyoto rat

2. LIST OF ORIGINAL PUBLICATIONS

I Kokkonen JO, Kuoppala A, Saarinen J, Lindstedt KA, Kovanen PT. Kallidin- and bradykinin-degrading pathways in human heart: degradation of kallidin by aminopeptidase M-like activity and bradykinin by neutral endopeptidase. Circulation 99:1984-90, 1999.

II Kuoppala A, Lindstedt KA, Saarinen J, Kovanen PT, Kokkonen JO. Inactivation of bradykinin by angiotensin-converting enzyme and by carboxypeptidase N in human plasma. Am J Physiol Heart Circ Physiol 278:H1069-74, 2000.

III Kuoppala A, Shiota N, Kokkonen JO, Liesmaa I, Kostner K, Mäyränpää M, Kovanen PT, Lindstedt KA. Down-regulation of cardioprotective bradykinin type-2 receptors in the left ventricle of patients with end-stage heart failure. J Am Coll Cardiol 40:119-25, 2002.

IV Kuoppala A, Shiota N, Lindstedt KA, Rysä J, Leskinen HK, Luodonpää M, Liesmaa L, Ruskoaho H, Kaaja R, Kovanen PT, Kokkonen JO. Expression of bradykinin receptors in pressure overload hypertrophy and heart failure. Submitted for publication.

3. REVIEW OF THE LITERATURE

1. Heart Failure

Heart failure (HF) is defined as a pathophysiological state in which the heart is unable to pump blood at a rate commensurate with the requirements of the metabolizing tissues, or when it can do so only with an elevated filling pressure. HF is usually, although not always, caused by a defect in myocardial contraction, i.e. by myocardial failure (Colucci and Brownwald, 2001).

HF is a common disorder in the western world. In the United States (no extensive data exist from Finland directly), 4.6 million persons, i.e. ~1.6% of the total population, are treated for HF, and 550,000 new cases of HF are diagnosed every year. Approximately 45,000 deaths are caused by HF annually and 260,000 deaths have HF as a contributing cause. The prevalence of HF increases with age, being 1-2% of persons aged 50-59 and 6-8% of individuals over the age of 75 years (Colucci and Brownwald, 2001). Thus, HF is a leading hospital discharge diagnosis in patients over 65 years old, and its prevalence is increasing, as shown by a 55% increase in the number of hospitalizations due to HF between the years 1985 and 1995 (Colucci and Brownwald, 2001).

Congestive heart failure (CHF) has a number of different underlying causes. In the Framingham Study (Ho *et al*., 1993), coronary heart disease (CHD) was the most important cause of CHF, accounting for approximately 55% of cases, followed by hypertension (25%) and valvular heart diseases (VHD) (16%). In addition to these major causes of CHF, other diseases, such as diabetes and hypertrophic cardiomyopathy, also contribute to the pathogenesis of this disorder.

Interestingly, as a result of the aggressive treatment of hypertension, the relative importance of these etiologies changed dramatically between the 1950s and the 1980s. In the 1950s, the most important cause of HF was hypertension, accounting for almost 50% of cases, while CHD accounted for 22% and VHD for 16% of cases. In the 1980s, the major cause of HF was CHD (67%), whereas hypertension was responsible for 25% and VHD for 10% of cases (Ho *et al*., 1993, Levy *et al*., 1996). In two Finnish patient groups with CHF, 50-60% of the patients had a diagnosis of CHD, 50-55% of hypertension, and 12-50% of VHD (Remes *et al*., 1992, Kupari *et al.,* 1997).

1.1. Pathogenesis of heart failure

Regardless of the underlying cause, the pathogenesis of HF is characterized by a number of structural and functional changes in the heart and in the circulatory physiology. The pathogenesis of HF is mostly that of a progressive phenomenon, in which the initial step seems to be a challenge to the function of the heart (either the systolic function, contractility, or the diastolic function, relaxation). This initial event may be induced by an acute incident, such as myocardial infarction (MI) myocarditis, or by a chronic disease, such as hypertension, diabetes, or VHD. The initial stimulus activates the compensatory mechanisms of the circulatory system, such as the beta-adrenergic system and the renin-angiotensin-aldosterone (RAS) system. In the short term, these compensatory mechanisms protect the heart by balancing the cardiac output with the demands. In the long term, however, the compensatory mechanisms

become deleterious to the myocardium, leading to loss of myocytes and formation of the extracellular matrix in excess (Colucci and Brownwald, 2001).

The most important structural change (remodelling) induced by the compensatory mechanisms is left ventricular hypertrophy (LVH)**.** Its induction is at least partly a result of activation of all the previous signaling cascades. LVH is characterized by two major components, myocyte hypertrophy and myocardial fibrosis, the latter mostly perivascular but also interstitial ((Weber *et al.*, 1988, Bing *et al.,* 1995), Conrad *et al.*, 1995). LVH affects the function of the heart in many ways: initially it compensates for the volume or the pressure overload by increasing either the size of the left ventricle or its capasity to produce force. In the long run however, LVH impairs the diastolic function of the heart by increasing the stiffness of the left ventricle working consecutively against the active phase of relaxation and the atrial filling (Ross and Braunwald, 1964, Lecarpentier *et al.*, 1987, Douglas *et al.*, 1989). Later, the stiffness produced by LVH will begin to interfere with the contraction of the left ventricle, so affecting the systolic function of the heart also. It seems very likely that some of the keys to the progressive nature of LVH and HF are the activated signal peptide systems. Several of these peptides have been shown to induce LVH when infused into animal hearts, and their inhibition has been a successful means of improving the otherwise very poor prognosis of HF patients. The most important of these signaling systems include the β-adrenergic system, the RAS, the endothelin system, and the inflammatory cytokines (Colucci and Brownwald, 2001).

The cardiovascularly deleterious signal peptide systems

In HF, the β-adrenergic system is activated. In failing myocardium, however, this system is tuned down, which results in loss of one compensatory system for acute incidents, while, in the peripheral circulation, activation of the β-adrenergic system causes vasoconstriction. This peripheral vasoconstriction increases the afterload and oxygen consumption of the heart, causing hypoperfusion and increased anaerobic metabolism in the tissues. The β-adrenergic system also promotes fibrosis, arrhythmias, and possibly necrosis and apoptosis, in addition to activating other signaling systems, such as the RAS (Floras *et al.*, 1993, Francis *et al.*, 1990). The RAS exerts effects similar to those of the β-adrenergic system, with the exception of the arrhythmias. In addition to these, it increases retention of salt and water and causes electrolytic disturbances (Timmermans *et al.*, 1993). Endothelins, through endothelin receptors, increase peripheral and pulmonary vasoconstriction and accelerate structural changes and apoptosis in the myocardium (Sam *et al.*, 1999, Ito *et al.*, 1991). Lastly, cytokines such as the tumor necrosis factor α (TNF-α), some interleukins and interferon γ (IFN-γ) decrease the contraction of the myocardium and induce LVH and the dilatation of the left ventricle. They may also possibly increase apoptosis (Finkel *et al.*, 1992, Palmer *et al.*, 1995. Bryant *et al.*, 1998).

The cardioprotective peptide systems

An increasing body of evidence indicates that, in addition to signaling systems which damage the myocardium, there are also protective systems opposing these deleterious effects. The systems most studied are: the kininogen-kallikrein-kinin (KKK) system and the natriuretic peptide system. A recently discovered addition to these is the

adrenomedullin system. The KKK system is described in greater detail in the following chapters.

There are three different atrial natriuretic peptides, types A, B and C, which exert their cardioprotective effects through the natriuretic peptide receptor A (A- and B-types) and the natriuretic peptide receptor B (C-type). These peptides produce vasodilatation, increase water and sodium secretion by the kidneys, decrease antidiuretic hormone (ADH) secretion and reduce thirst. They also oppose endothelin, RAS, and the β-adrenergic-system (Struthers *et al.*, 1994. Moe *et al.*, 1993, Wada *et al.*, 1994). In addition BNP has a direct anti-fibrotic effect in the myocardium (Tamura *et al.*, 2000). Thus, by opposing the harmful effects of these systems, the atrial natriuretic peptides directly provide cardioprotection (Calderone *et al.*, 1994).

Adrenomedullin exerts effects similar to those of the atrial natriuretic peptides, i.e., it causes vasodilatation and growth inhibition in the myocardium and opposes endothelin, while increasing the excretion of sodium and water (Hinson *et al.,* 2000).

On the basis of these findings, it has been hypothesized that a balance exists between the harmful signaling systems and the cardioprotective systems, which stabilize the homeostasis of the normal heart. In the beginning of the pathogenesis of HF this equilibrium is disturbed, resulting in the onset of LVH, followed by impaired function of the heart, and finally leading to HF. Figure 1 presents the theory of the signaling systems and their balance.

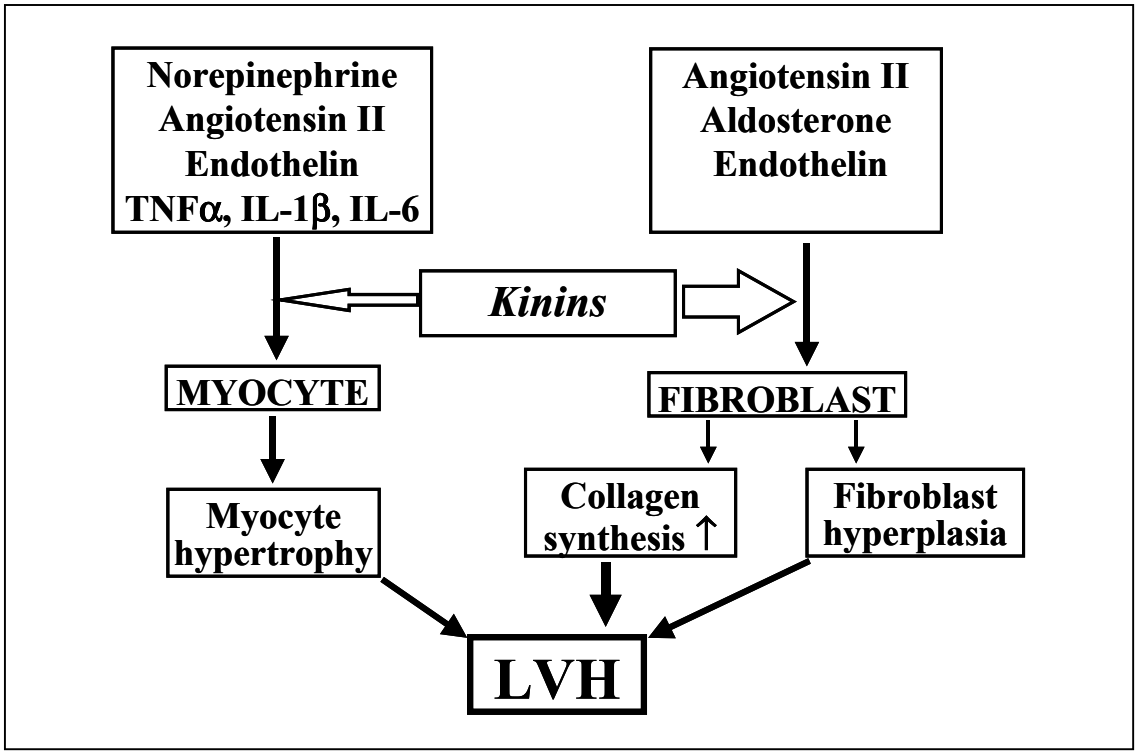

Figure 1. The cardiovascularly active peptide systems.

2. The kininogen-kallikrein-kinin system

The history of the (brady)kinin system started when two French surgeons observed a transient fall in blood pressure in a patient after an intravenous injection of fractions extracted from human urine (Abelous *et al*., 1909). In 1925 surgeon Emil-Karl Frey similarly observed a considerable reduction in blood pressure when he injected the urine of humans into dogs. He attributed this effect to a substance with potential biological functions (Frey, 1926, Frey and Kraut, 1926). ”It is a substance that probably originates from several organs, is eliminated by the kidneys and has a pronounced cardioactive and vasoactive effect; a substance that is assigned the role of a hormone in the organism”. This F-substance was later termed kallikrein (KLK) (Kraut *et al*., 1930). Ten years later, Eugen Werle found that KLK (tissue kallikrein) is a proteolytic enzyme, which liberates the biologically highly active basic polypeptide kallidin (KD) from a plasma protein, kallidinogen or kininogen (Werle *et al*., 1937). Werle also observed the degradation of kinins by ”kininases” and identified these as peptidases (Werle and Grunz, 1939).
In 1949, Rocha e Silva (1949) discovered that trypsin, when incubated with blood, releases an agent that contracts the guinea-pig ileum; the response of this tissue develops slowly, so the authors called the agent ”bradykinin” (brady=slow). Later he purified bradykinin (BK) and determined it to be a peptide (Rocha e Silva, 1955, Andrade and Rocha e Silva, 1956). The exact sequence of BK was designated by Swiss chemists and the nonapeptide was chemically synthesized (Boissonnas *et al*., 1960, Erdös 1970).

2.1. Bradykinin and kallidin

The two peptides usually referred to as kinins are BK and KD or lysyl-BK. BK is a nonapeptide that can be found in basically all secretions of the body, i.e. urine, saliva and sweat, but also in feces and in several tissues, such as the heart, vasculature, blood, kidneys, liver, colon, reproductive organs, skin, brain, lungs, small intestine, brown adipose tissue and adrenal glands (Martinez *et al*., 1981, Campbell DJ *et al*., 1993, Hibino *et al*., 1994, Patel S *et al*., 1999, Schremmer-Danninger *et al*., 1999, Madeddu *et al*., 2001a, Meneton *et al*., 2001). BK is produced by plasma KLK (pKLK), whereas KD is produced by tissue KLK. BK can also be produced from KD by several aminopeptidases through cleavage of the aminoterminal lysine.

KD has been shown to exist in the heart, urine (kidney), and circulation (Campbell *et al*., 1999a, Duncan *et al*., 2000). Since tissue KLK and LMW kininogen are both expressed in such a variety of tissues, it may be hypothesized that all these tissues also contain KD. However, the low KD concentrations in blood (<1 pM) and tissues (0.5 and 4 pM) suggest that most of the KD is rapidly degraded into BK (Campbell *et al*., 1999a).

Other important kinin fragments

In addition to BK and KD, there are at least two other kinin fragments des-arg^{9}-BK, or BK-(1-8) and des-arg^{10}-KD or KD-(1-9) that can interact with kinin receptors. These two des-arg fractions of kinins are agonists for the type 1 BK receptor (BK-1R). Interestingly, in humans and dogs des-arg^{10}-KD is a stimulator of the BK-1R, being several fold more potent than des-arg^{9}-BK, whereas in rodents, the opposite

seems to be the case (McLean *et al*., 2000). Finally, BK-(1-7) seems to be an inactive degradation product, whereas BK-(1-5) may be involved in the coagulation system (Hasan *et al*., 1996). Figure 2 depicts the structure of kinins.

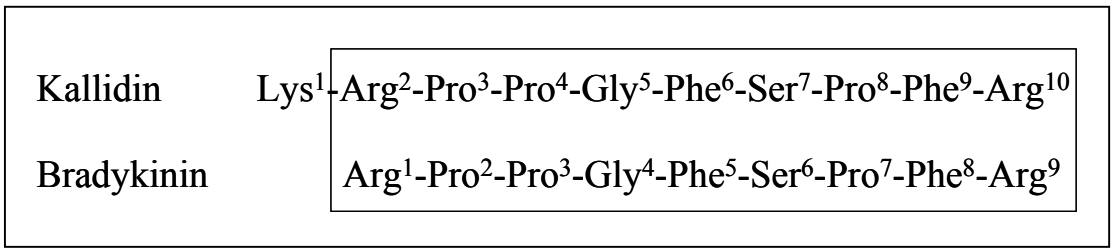

Figure 2. Structure of kinins.

2.2. Regulation of kinin concentration

The concentrations of kinins in the circulation, in the vascular tree and in the interstitium of the heart are regulated by the levels of kinin formation and degradation. Kinins are formed and degraded by reactions involving several different enzymes. The relative importance of the specific enzymes involved in the process may differ in each tissue, resulting in different local profiles of kinin fragments.

2.2.1. Formation of kinins from kininogens

In the human KKK system, kinins are formed from kininogens. There are two types of kininogens, high-molecular-weight (HMW) (88 to 120kDa) and low-molecular-weight (LMW) (50 to 68kDa) type kininogens, which are coded by a single gene via alternative splicing. The human kininogen gene, which maps to chromosome 3q26-qter, i.e., in the vicinity of two closely related members of the cystatin superfamily, the alpha-2-HS-glycoprotein and the histidine-rich glycoprotein, is 27-kb long and contains 11 exons (Kitamura *et al*., 1985). For the production of HMW kininogen, the first ten exons are translated, and for LMW kininogen the first nine, the BK-containing start of the 10^{th} and the 11^{th} exon (Müller-Esterl *et al*., 1986). The use of antibodies against the different domains of the kininogen molecule has led to the conclusion that the kininogen molecule has 6 domains: domain 1 for calcium binding, domains 2 and 3 for inhibition of cysteine proteinases such as cathepsins, domain 5 for binding to surfaces such as the endothelium, domain 6 for binding factor XI, and domain 4, which contains the kinin entity (Weisel *et al*., 1994).
Both kininogens were traditionally thought to be produced by the liver, but recently several other tissues have also been shown to produce kininogens. Thus, high expression of HMW kininogen mRNA has been shown in the liver, with some also present in the skin, lungs, and testes, but not in the heart. LMW kininogen mRNA is present in the lungs, brain and heart, and specifically in the cardiac myocytes (Yayama K *et al*., 2000 and 2001, Neth *et al*., 2001).

2.2.1.1. Kallikreins

Kinins can be formed from either HMW or LMW kininogen by enzymes called kininogenases or KLKs. Some other enzymes have also been suggested to have

kininogenase activity, but of these only the combination of neutrophil elastase and mast cell tryptase has been shown to produce BK under inflammatory conditions (Kozik *et al.*, 1998). In the tissue KLK gene family, there are at least 13 different ”kallikreins”, but at present only one of these is considered to be a true kininogenase: tKLK (Kallikrein 1, kidney kallikrein, true kallikrein). In addition, an enzyme that is genetically unrelated to tKLK, i.e., pKLK (prekallikrein, Kallikrein B1), also has potent kininogenase activity.

Tissue kallikrein

Tissue kallikrein (tKLK) is strongly expressed in the salivary glands, kidneys, colon and pancreas, and weakly expressed in blood vessels, heart, sweat glands, intestine, central nervous system, neutrophils, uterus, prostate, testes, breast and placenta (Bhoola *et al.*, 1992, Clements *et al.*, 1997, Harvey *et al.*, 2000, Meneton *et al.*, 2001). The tKLK gene has been localized to chromosome 19, and more specifically to 19q13.3-13.4 (Evans *et al.*, 1988, Sutherland *et al.*, 1988). The tKLK gene has been considered to have only one transcript, although some mRNA variants have been described (Evans *et al.,* 1988, Rae *et al.*, 1999). None of these variants have yet been shown to be translated, suggesting that they may be nonfunctional. TKLK is secreted as a preproenzyme and is proteolytically processed into a proenzyme by removal of the 17-amino-acid signal peptide. The proenzyme is further activated by the cleavage of a 7-amino-acid activation peptide. The activation of tKLK may occur by autoactivation, cleavage by other members of the KLK protease family or possibly by some other proteases (Yousef *et al.*, 2001).
The main substrate for tKLK has been considered to be LMW kininogen (Hilgenfeldt *et al.*, 1998), but tKLK can also produce kinin from HMW kininogen (Colman *et al.*, 1997). Regardless of the type of kininogen being cleaved, the peptide generated is KD. Apart from kininogens, tKLK may also have other substrates, such as the beta nerve factor (Bothwell *et al.*, 1979). In addition, tKLK has been shown to cleave proinsulin, low density lipoprotein, the precursor of the natriuretic peptide type A, prorenin, vasoactive intestinal peptide, procollagenase, and angiotensinogen. However, on the basis of the Km values and other enzymatic parameters of the enzyme on other substrates, it has been suggested that the primary effect of tKLK is *in vivo* kinin formation (Bhoola *et al.*, 1992).

TKLK seems to be an effective kininogenase in the interstitium of different tissues and, probably has its impact in such organs as the kidney and heart directly on the myocardial parenchymal cells through their BK receptors (Bhoola *et al.*, 1992). In support of this Meneton *et al.* (2001) showed that the tKLK knockout mouse has a four-fold lower BK concentration than its control in the kidney. However knocking out tKLK decreased the heart tissue kinin concentrations by only 25%. The kinin generating capacities of tissue homogenates were decreased 30 to 500-fold in the kidney, colon, salivary glands and pancreas, but no difference could be found in the heart tissue.

Plasma kallikrein

The expression of the plasma kallikrein (pKLK) gene seems to be highest in the liver, but it has also been shown to be expressed in many tissues, such as the pancreas, kidney and heart, and by several different cell types (Hermann *et al.*, 1999, Neth *et*

al., 2001). PKLK is decreased during liver diseases, supporting the view that the liver is the most important pKLK-producing organ (Fisher *et al.*, 1982). The gene of pKLK has been localized to chromosome 4, and more specifically to 4q34-35 (Beaubien *et al.*, 1991, Goold *et al.*, 1993). The mRNA codes for a 371 amino acid heavy chain and a 248 amino acid light chain and the form of the folded protein has four groups of 90-91 amino acids arranged in so-called apple domains (Chung *et al.*, 1986, McMullen *et al.*, 1991).

The activation of pKLK *in vivo* is a cascade of events. After an initial trigger, HMW kininogen attaches to the endothelium and a multiprotein receptor for prekallikrein assembles on top of it. Binding of prekallikrein to HMW kininogen leads to activation of pKLK and cleavage of BK from the HMW kininogen, followed by separation of pKLK from the HMW kininogen (Motta *et al.*, 1998). It has also been shown that prekallikrein can activate itself *in vitro* (Burger *et al.*, 1986). Although HMW kininogen is considered to be the best, if not the only, substrate for pKLK (Hilgenfeldt *et al.*, 1998), pKLK can also generate BK from LMW kininogen (Colman *et al.*, 1997). Other substrates for pKLK, apart from kininogens, include factor XII and prourokinase (Ichinose *et al.*, 1986, Hauert *et al.*, 1989).

Relative importance of tKLK and pKLK in BK formation in the heart

Both tKLK and pKLK are found in the heart tissue. Since they have the same end product, BK and the turnover of KD to BK is very high (therefore direct KD measurements do not seem very reliable) it is difficult to determine, which enzyme is more important for kinin formation in the heart. Logically it could be supposed that tKLK is responsible for kinin formation in the interstitium and pKLK in the vasculature. The levels of BK measured in the heart tissue are highest in the myocardium (measured from whole tissue pieces), ten-fold lower in the venous blood and lowest in the arterial blood (Campbell DJ *et al.*, 1993, Duncan *et al.*, 2000), suggesting that kinins may flow from the tissues into the blood and that the dominating kinin forming enzyme in the heart would be tKLK.
However, there is the controversial result of Meneton *et al.* (2001) on kinin formation in the heart of the tKLK-KO mouse.

These seemingly discrepant findings may be explained as follows:
PKLK acts locally at the luminal surface of the endothelium and therefore the highest concentration of BK produced by pKLK is that by the endothelial surface inside the vessel (also the location of the BK-2R). Moreover the half life of BK is very short (10-15 seconds in the plasma alone) and, because the myocardial demand for oxygen is so great, the blood passes through the myocardial vessels quickly. Considering the high rate of flow and short half life of BK in the plasma, it seems likely that the concentration of BK is several-fold higher at the capillary endothelial surface than in the venous blood. It may well be even higher than the kinin concentration in the interstitium and this would mean that, somewhat paradoxically, the interstitial kinins are mostly produced by pKLK, and not by tKLK .

The role of the endothelium and pKLK in BK formation is further sustained by a report showing that after MI, the outflow of BK is increased, but stops when the endothelium is removed (Linz *et al.*, 1994). In addition, it has been shown that in Brown-Norway-Katholiek rats, which lack the HMW kininogen but have some LMW

kininogen left (HMW type kininogen being the substrate for pKLK and LMW type for tKLK), the cardioprotective effects of BK on MI do not function, suggesting that the dominant BK producing system in the heart is the pKLK-HMW kininogen pathway (Yang XP *et al.*, 1997b).

2.2.2. Degradation of kinins

The enzymes responsible for the degradation of the receptor-active kinin peptides are called kininases. Since the discovery of kinins, kininases have been a target for active research. The reason for this relates to three facts: kinins were known to exert important biological activity, one way to control their activity was clearly degradation and the methodology for enzymological studies of kinins was already in general use when kinins were discovered.

Kininases

Kininases cleave kinins at either their aminoterminal or their carboxyterminal end. Figure 3 shows the major enzymes capable of degrading kinins. Although kininases with carboxyterminal cleavage specificity seem to dominate there are two major kininases capable of aminoterminal cleavage of kinins.

Firstly, aminopeptidase M (APM) can degrade KD, a kinin produced by tKLK, into BK (Wolfrum *et al.*, 1999). However, APM does not inactivate the kinins, since both KD and BK are agonists for the kinin receptors. Secondly, aminopeptidase P (APP) can cleave the first amino acid of BK yielding BK-(2-9). However, APP-mediated kinin degradation seems to be relevant only in rats, while in other species it is much less effective (Chen X *et al.*, 1991).

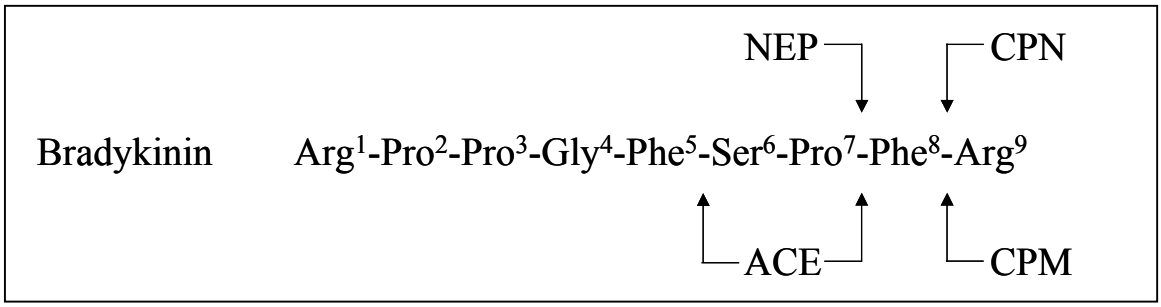

Figure 3. The major enzymes capable of degrading kinins.

There are four major enzymes responsible for the carboxyterminal degradation of kinins (Erdös *et al.*, 1990). The angiotensin-converting enzyme (ACE) and the carboxypeptidases N and M (CPN, CPM), were discovered as long ago as in the 1960s, whereas the neutral endopeptidase (NEP, also enkephalinase or neprilysin), was found in 1973 (Camargo *et al.*, 1973). These kininases can be divided into two groups on the basis of their enzymology, kininases I: CPN and CPM, and kininases II: ACE and NEP. The terms kininase I and kininase II were originally used for CPN and ACE, respectively. Enzymes of kininase type I cleave the carboxyterminal arginine from either BK or KD to yield des-Arg^9-BK or des-Arg^{10}-KD (BK-(1-8) or KD-(1-9), whereas enzymes of kininase type II cleave the dipeptide Phe^8-Arg^9 from both BK and KD to yield BK-(1-7) and KD-(1-8). These are further cleaved by ACE to yield

BK-(1-5) and KD-(1-6). Finally, when the enzymology of kininases is studied and interpreted, one must take into account that there are significant differences between species in their relative kininase activities (Decarie *et al.*, 1996).

Degradation of kinins in the circulation.

The results of studies on the enzymatic degradation of BK in human plasma or serum have been controversial. In one report (Odya *et al.*, 1983), the major kinin peptide in human plasma was found to be BK-(1-8), suggesting that BK was mostly degraded by CPN. In studies by Marceau *et al*. (1981) and Sheikh and Kaplan (Sheikh and Kaplan, 1989), incubation of plasma with synthetic BK revealed that the major BK-degrading enzyme was CPN, with ACE playing only a minor role. In contrast, in a recent report, in which a chemiluminescent enzyme immunoassay was used to measure the changes in BK concentration, Decarie *et al.* (1996) suggested that about 2/3rds of the BK-degrading activity in human serum and plasma was due to ACE activity.

Degradation of kinins in the heart interstitium.

ACE is generally thought to be the most important enzyme responsible for the degradation of BK in the heart (Margolius *et al.*, 1995). This notion is supported by several observations. 1. The cardioprotective effects of the kinins appear to be linked with the activity of the ACE (EC 3.4.15.1), since BK receptor blockers partially block many of the beneficial effects of ACE inhibitors (ACEi), for example, on heart remodeling in dog and rat models (McDonald *et al.,* 1995, Liu YH *et al.*, 1997, Wollert *et al.*, 1997). 2. *In vitro* experiments have demonstrated that purified ACE readily degrades BK to BK-(1-7) and further to BK-(1-5) (Dorer *et al.*, 1974). 3. Inhibition of ACE has been shown to increase the outflow of BK from isolated perfused rat hearts (Baumgarten *et al.*, 1993).

Recent findings have also suggested that, in addition to their inhibitory effect on the degradation of kinins, part of the beneficial effects of ACEis can be attributed to their direct effects on BK receptors (Hecker *et al.*, 1997, Minshall *et al.*, 1997b). Furthermore, by measuring kinin levels directly in rat heart tissue, it has been shown that ACEi does not affect the BK levels, strongly suggesting that enzymes other than ACE may be responsible for BK degradation in the myocardium (Campbell DJ *et al.*, 1994). However, all the current information relies on animal models and no information is available on kinin metabolism in human heart tissue either in health or disease.

2.3. BK Receptors

To date, kinins have been shown to interact with two specific receptor molecules, BK type 1 (BK-1R) and type 2 (BK-2R) receptor, although another one or two have been proposed to exist (Llona *et al.*, 1987, Rifo *et al.*, 1987). BK-2R has been under very intense research for the last twenty years, whereas BK-1R has received more attention mainly during the last five years.
Although the existence of a BK receptor was already suspected in the early 1970s (Watson, 1970, Vogel *et al.*, 1971, Damas and Cession-Fossion, 1973), the first attempt to define and analyze the BK receptors more systematically, on the basis of

the receptor theory by Ariens (Ariens, 1964), was made by the group of Regoli and Barabe in 1974 (Barabe *et al.*, 1975). In 1977, the group of Regolis first described two different types of BK receptors in the rabbit (Barabe *et al.*, 1977, Regoli *et al.*, 1977), and in 1978 they showed that both types of BK receptors coexisted in rabbit veins, one (BK-2R) being stably expressed and the other (BK-1R) being inducible (Regoli *et al.*, 1978).
The first actual myocardial BK receptors (later shown to be BK-2R) were described in the nerve endings of heart tissue from dogs in1976 (Staszewka-Barczak *et al.*, 1976) and in the atrium of guinea-pigs (Iven *et al.* 1980), and much later in the myocytes (Minshall *et al.*, 1995). Since the discovery of kinin receptors, a growing body of evidence has indicated their involvement in several physiological functions and diseases of the heart.

2.3.1. BK-2R

BK-2R was initially characterized in the rat uterus, rabbit aorta and jugular vein by Barabe, Regoli and Marceau together with co-workers in1975-1979 (Barabe *et al.*, 1975, Barabe *et al.*, 1977, Regoli *et al.*, 1977, Barabe *et al.*, 1979). Subsequently BK receptor research concentrated on pharmacological studies, and the receptor molecule was cloned from a rat smooth muscle cell (SMC) library by McEachern *et al*. (1991) and from human lung fibroblasts (fibroblasts) by Hess *et al.* (1992). The human cDNA clone encoded a 364-amino acid protein, later shown to have a molecular mass of 41 kDa, which had the characteristics of a 7-transmembrane domain G protein-coupled receptor (Hess *et al.,* 1992, Powell *et al.*, 1993). The predicted human amino acid sequence showed 81% identity with the rat smooth muscle BK-2R.
It was concluded that the BK-2R is encoded in humans by a single copy gene, which is located in chromosome 14 (Powell *et al.*, 1993) and more specifically at 14q32 (Ma *et al.*, 1994, Kammerer *et al.*, 1995). The gene structure is arranged in three exons and two introns and codes for a single transcript of 4kb. The coding region for the mature protein is located in the third exon (Ma *et al.*, 1994).

2.3.1.1. Physiological effects of BK-2R

BK-2R exerts several different effects on the physiology of a number of different tissues. In the vasculature, BK-2R signaling can lead to vasoconstriction or vasodilatation, and in the parenchymal tissues it can cause either stimulation or inhibition of growth, when stimulated on the surface of either SMCs or endothelial cells (ECs), respectively (Dixon *et al.*, 1994a, Rosenkrantz *et al.*, 1999, Douillet *et al.*, 2000, Kamei *et al.*, 2000). BK-2R has been shown to be antiarrhythmic in the heart (Linz *et al.*, 1986) and antithrombotic in the vasculature (Brown *et al.*, 2000), and to reduce infarct size and precondition the heart against ischemic events (Vegh *et al.*, 1991, Yoshida *et al.*, 2000). In HF, BK-2R also seems to improve the myocardial use of oxygen, possibly by attenuating the endothelial dysfunction (Jeserich *et al.*, 1995, Pittis *et al*., 2000).

In addition to the cardiovascular system, BK-2Rs have been shown to affect several other systems also. In diabetes, BK-2R affects the glucose metabolism both directly and through interaction with insulin (Rosenthal *et al.*, 1997, Kudoh *et al.*, 2000b). In the alimentary tract, kinins and kinin receptors have been found to affect the SMCs of the duodenum, ileum and cecum, causing either contraction or relaxation (Antonio

1968, Hall and Bonta 1973, Gater *et al.*, 1985). In the respiratory tract kinins have been implicated in Cl^- secretion and bronchoconstriction. As a result of this finding, BK receptors have been implicated in the pathogenesis of asthma (Collier 1962, Leikauf *et al.*, 1985, Barnes *et al.*, 1988). Kinins also seem to affect the functions of reproductive organs and the bladder, by inducing smooth muscle contraction in the vas deferens, in the uterus, and in the bladder (Whalley, 1978, Marceau *et al.*, 1980, Llona *et al.*, 1987).

Lastly, BK-2R has been implicated in the physiology and pathophysiology of inflammation, pain, and hyperalgesia (Armstrong *et al.*, 1952, Schachter *et al.*, 1987, Steranka *et al.*, 1988). BK is one of the substances that produce strong pain, an effect mediated via the BK-2Rs (Whalley *et al.*, 1987). In addition, kinin-mediated BK-2R activation can induce inflammatory responses either directly or by interacting with other inflammatory mediators, such as cytokines, to amplify inflammatory effects (Devillier *et al.*, 1985, Schachter *et al.*, 1987, Burch *et al.*, 1988).

2.3.1.2. The regulation of BK-2R

BK-2R upregulation

Since BK-mediated stimulation of BK-2Rs does not affect the BK levels, it seems evident that no regulatory short feed-back loop exists between kinins and BK-2Rs (Campbell DJ *et al.*, 1999c). It has also been shown that endogenous kinins do not exert negative feedback regulation on the BK- 2Rs, not directly at the level of receptor expression (Marceau *et al.*, 1999, Tschöpe *et al.*, 1999a and 1999b) or through cellular regulation of receptor endocytosis (Bachvarov *et al.*, 2001, Marceau *et al.*, 2001). On the contrary, BK itself has been shown to upregulate expression of a reporter gene, driven by the BK-2R promoter (Pesquero *et al.*, 1996).

Inflammatory signals. Increasing evidence indicates a role for inflammatory signals that use the Ras oncogene system in the induction of BK-2R expression (Parries *et al.*, 1987, Downward *et al.*, 1988, Ruggiero *et al.*, 1989, Pesquero *et al.*, 1996). So far, two receptor systems using the Ras pathway for the induction of BK-2R have been described: interleukin 1β (IL-1β), and platelet-derived growth factor (PDGF) (Dixon *et al.*, 1996, Phagoo *et al.*, 2000, Yang CM *et al.*, 2001). IL-1β induces BK-2R expression at least partly through the Ras-Raf-MEK-MAPK pathway (Yang CM *et al.*, 2001). In bronchial SMC, IL-1β also uses prostaglandin E_2 (PGE_2) through the cAMP dependent pathway (both PGE2 and cAMP also independently stimulate BK-2R expression), phospholipase A_2 (PLA_2) phosphorylation and the p38 MAP kinase pathway (Dixon *et al.*, 1994b, Costenbader *et al.*, 1997, Castano *et al.*, 1998, Schmidlin *et al.*, 1998b, Schmidlin *et al.*, 2000). Glucocorticoids can inhibit the induction of BK-2Rs by IL-1β (Schmidlin *et al.*, 1998a). On the other hand, glucocorticoids may also upregulate the BK-2Rs in tracheal SMCs independently of IL-1β (Scherrer *et al.*, 1999). In addition to IL-1β, other inflammatory mediators and effector molecules, such as IFNγ and the p53 tumor suppressor gene, have been shown to upregulate BK-2R, (Lung *et al.*, 1998, Saifudeen *et al.*, 2000).

Components of the KKK. In a mouse model, knocking out $G\alpha i_2$, one of the two major Gα proteins in the BK-2R signaling, seems to upregulate BK-2R expression,

suggesting a feedback link between BK-2Rs and the G-proteins mediating the receptor effects (Mattera *et al.*, 1998).

Direct effects of ACE inhibitors on BK-2R activity and function

It has previously been suggessted that ACEis are more effective than AT_1-antagonists in preventing the progression and complications of HF (Rouleau *et al.*, 2000, Packer *et al.*, 2002) This additional effect has been related to the increase in kinin concentrations due to inhibition of kinin degradation. Since AT_1-antagonists have been shown to affect the KKK system via AT_2 receptor activation, other factors have also been considered. One hypothesis is that ACEis, in addition to inhibiting kinin breakdown, may affect BK-2Rs directly (Erdös *et al.*, 2001). This was first suggested after the finding that other peptides, in addition to ACEi, potentiated BK-2R function (Tewksbury *et al.*, 1968, Vogel *et al.*, 1970, Ufkes *et al.*, 1977, Rubin *et al.*, 1978, Chi *et al.*, 1985). The apparent possibility of interactions between ACEis and BK-2Rs has been confirmed as very likely (Benzing *et al.*, 1999, Danser *et al.*, 2000).

In addition, Hecker *et al. (1994 and 1996)* showed that inhibition of ACE with high concentrations of ACE substrates did not mimick the ACEi-mediated effects on the KKK system. Even in the presence of ACE-resistant BK-2R agonists, the ACEis still exerted an enhancing effect on BK-2R signaling (Marcic *et al.*, 1999). Furthermore in the guinea pig ileum the effects of ACEi on BK degradation occur after 12-15 minutes, whereas the effects on BK-2R signaling are seen within seconds (Minshall *et al.*, 2000). In addition, substitution of the transmembrane part of ACE with a different anchor blunted the effect of ACEi on BK-2R signaling (Marcic *et al.*, 2000) and agents blocking only one of the two active sites of ACE potentiated BK independently of blocking peptide metabolism by inducing crosstalk between ACE and the receptor (Marcic *et al.*, 1999).

Although ACEi seems to cause an increase in the activity of BK-2Rs, opposite results concerning the direct influence of ACEis on BK-2Rs have also been published, claiming that the ACEi effects depend solely on the inhibition of BK degradation (Dendorfer *et al.*, 2000 and 2001a).

BK-2R downregulation and inactivation

The only factor shown to reduce the BK-2R level is the tumor necrosis factor TNF-α (Sawutz *et al.*, 1992, Haddad *et al.*, 2000). However, it has been shown that a temporary decrease in the number of active receptors on the plasma membrane can be achieved by desensitization and internalization through receptor phosphorylation, and by dimerization via the aminoterminal end of the receptor. In addition to kinins, receptor desensitization may also be achieved by the BK-2R antagonist HOE-140 or by an increase in intracellular Na^+-ions. In fact, an increase in Na^+ decreases the BK-2R levels on the cell surface, whereas a decrease in Na^+ increases the level of the receptors (Quitterer *et al.*, 1996, Pizard *et al.*, 1998, AbdAlla *et al.*, 1999, Houle *et al.*, 2000a). In addition, stimulation of BK-2Rs with agonists reduces the affinity of the receptor towards the ligand, thereby requiring higher ligand concentrations for stimulation (Dendorfer *et al.*, 2000). Internalization of the BK-2R also seems to be short-lived, since, when the agonists are withdrawn, complete restoration of the receptor follows in 30 minutes (Windischhofer and Leis, 1997). It also appears that

the BK-2Rs are almost completely recycled and, therefore, do not involve a permanent downregulation of the receptor during the process (Bachvarov *et al.*, 2001, Lamb *et al.*, 2001). In addition, nitric oxide (NO), the major second messenger molecule downstream of BK-2Rs, seems to exhibit a negative feedback loop by selectively inhibiting the Gi and Gq subforms of the G-proteins and the ligand-binding ability of BK-2R via a cGMP-dependent pathway (Miyamoto *et al.*, 1997).

Polymorphisms of BK-2R and ACE genes

The BK-2R gene has been shown to contain several polymorphisms both in the promoter region and in the exons. For example, point mutations at bases –58 (T/C) and -412 (C/G) in the promoter (Braun *et al.*, 1996), and a point mutation at +181 (C/T) in the exon 2 (Braun *et al.*, 1995, Houle *et al.*, 2000b) have been described. In addition, a 9-basepair deletion (+9/-9) at +21-29 in exon 1 was found to be associated with with expression of a larger amount of mRNA by the deleted allele (Lung *et al.*, 1997).
The only polymorphism in the ACE gene that has been linked to the kinin system is the insertion/deletion (I/D) polymorphism in which the D-allele is associated with an increase in ACE activity (Murphey *et al.*, 2000).

Polymorphism –58T/C. It has recently been shown that the –58T/C BK-2R polymorphism, and more precisely the C allele thereof, is associated with hypertension in three independent populations, i.e., Chinese, Japanese and African-Americans (Mukae *et al.*, 1999, Gainer *et al.*, 2000, Wang B *et al.*, 2001).

Polymorphism +9/-9. The most important BK-2R polymorphism seems to be the +9/-9 deletion in exon 1. In 1997 this polymorphism, namely insertion, with stronger expression of the protein (+9/-9), was first suggested to have an impact in the pathology of a disease, angioedema (Lung *et al.*, 1997). However, lack of this deletion (with lower expression of BK-2R) in conjunction with ACE D polymorphism (with higher ACE activity and therefore lower BK levels) is associated with increased fibrosis in the development of LVH, strongly suggesting an opposite role for BK-2R in the development of LVH (Brull *et al.*, 2001). Previous studies by these authors showed that an AT_1-antagonist did not affect the development of hypertrophy in persons with the D allele, further emphasizing the importance of kinins rather than Ang II as substrates of ACE and effector molecules of ACE in the pathology of LVH (Myerson *et al.*, 2001).

ACE insertion polymorphism. The ACE I/D polymorphism, and in particular the D allele, also seems to have an impact on endothelial function, being associated with blunting of stimulated release of endothelial NO in young healthy adults (Butler *et al.*, 1999). The D-allele has also been shown to cause significant differences in the vasodilatory response of vessels to BK in both forearm and femoral vessels (van Dijk *et al.*, 2000, Arcaro *et al.*, 2001). This polymorphism also affects the coronary arteries and has been shown to affect blacks more than whites (Prasad *et al.*, 2000, Gainer *et al.*, 2001).
Underlining the importance of this, the D allele has been shown to be enriched in populations of patients from Slovenia and Japan with MI and CHD (Cambien *et al.*, 1992, Peterlin *et al.*, 2000, Aoki *et al.*, 2001).

2.3.1.3. Intracellular cascades and second messengers of BK-2R

Initial events

Stimulation of BK-2Rs with BK triggers several second messenger cascades in the major myocardial cells, i.e. ECs, cardiomyocytes (CMC), fibroblast and SMCs. Figure 4 gives a schematic representation of the intracellular signaling cascades of the BK-2R. The BK receptors belong to the family of so-called G-protein-coupled receptors (GPCR) and BK-2R especially seems to be consistently linked to two G-proteins: $G\alpha_{i2}$ and $G\alpha_q$ (Gutowski *et al.*, 1991, Liao *et al.*, 1993, Busse *et al.*, 1996). When the ligand attaches to BK-2R, the G-protein cascades are activated and result in the activation of phospholipases, i.e. PLC, PLA_2 and PLD (Revtyak *et al.*, 1990, Gutowski *et al.*, 1991, Minshall *et al.*, 1995, Zugaza *et al.*, 1997, Banno *et al.*, 1999). Activation of phospholipase C (PLC) results in increases in inositoltriphosphate (IP_3) and diacylglycerol (Francell *et al.*, 1987) and activation of PLA_2 in the formation of IP_3 and activation of the arachidonic acid/prostaglandin pathway, producing PGE_2 and prostacyclin (PGI_2) (Burch *et al.*, 1987, Gallagher *et al.*, 1998, Saunders *et al.*, 1999, Yamasaki *et al.*, 2000). Activation of the two phospholipase cascades in concert cause a biphasic increase in intracellular Ca^{2+}, which together with diacylglycerol, leads to the translocation of PKC (Blaukat *et al.*, 1996). Phospholipase D (PLD) is activated through PKCα and PKCδ and possibly by an unidentified tyrosine kinase (Vasta *et al.*, 1998, Lee *et al.*, 2000, Levine *et al.*, 2000, Meacci *et al.*, 2000). Activation of PLD leads to increases in phosphatidic acid (PA) and diacylglycerol, which further increase the level of intracellular Ca^{2+} (Angel *et al.*, 1994, Walter *et al.*, 2000). Indeed, all phospholipases in the downstream signaling cascade of BK-2R activation increase both the Ca^{2+} level and the prostaglandin synthesis in the target cells, suggesting that these key mechanisms are the main mediators of the BK-2R-mediated effects.

Most importantly, the increase in intracellular calcium results in activation of endothelial nitric oxide synthase (eNOS). ENOS is the enzyme which produces NO, not only in the ECs, but also in the myocytes (Ritchie *et al.*, 1998b, Matoba *et al.*, 1999). In the plasmalemmal caveolae, eNOS seems to be co-localized with many of its coeffectors, such as G-proteins I and q, Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway kinases (tyk2, STAT3), and the whole MAPK pathway (Belhassen *et al.*, 1997, Liu P *et al.*, 1997, Marrero *et al.*, 1999, Ju *et al.*, 2000, Oh *et al.*, 2001), and PLD (Meacci *et al.*, 2000) in addition to the structural proteins of the caveolae: caveolins 1 and 3 (Feron *et al.*, 2001).

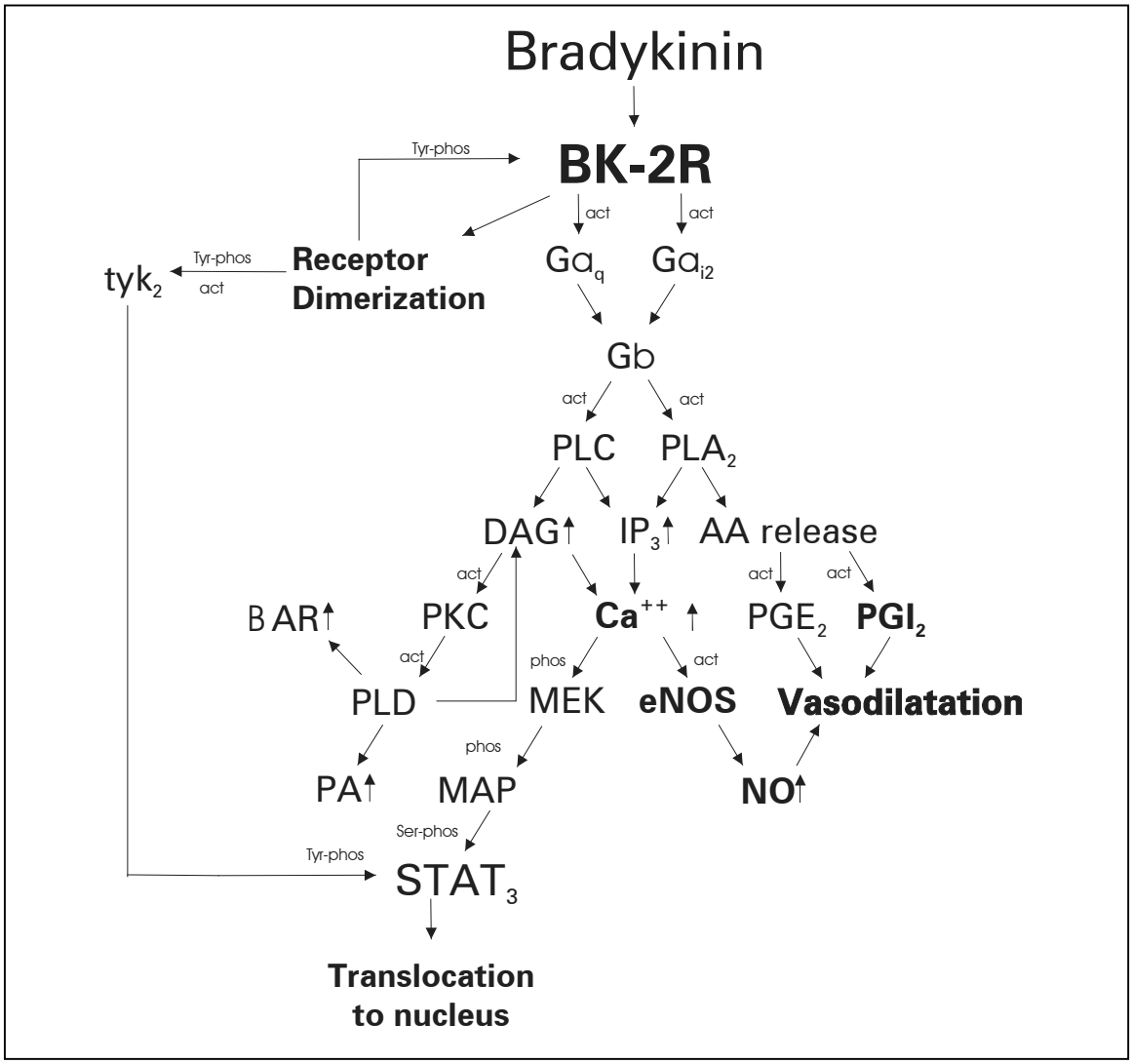

Figure 4. The intracellular signalling cascades of the BK-2R in endothelial cells.

Plasmalemmal caveolae

The caveolae are specialized plasmalemmal lipid microenvironments that seem to have two main functions. Firstly, by bringing different signaling molecules together in close proximity they act as signaling platforms, producing a microenvironment for the tight regulation and concerted function of receptors and their intracellular signaling cascades. Secondly, they also act as intracellular trafficing modules. These two functions are closely related and complementary. The structural components of caveolae consist of proteins, the caveolins, of which there are different types for different types of cells (e.g. caveolin 1 for ECs and caveolin 3 for myocytes) (Feron *et al.*, 2001).

Before stimulation, BK-2Rs seem to be located partly in the caveolae and partly on the ”regular” plasma membrane, (de Weerd *et al.*, 1997, Ju *et al.*, 2000). However, after stimulation of BK-2Rs with agonists, the receptors move to the caveolae (de Weerd *et al.*, 1997). In addition, the receptors are phosphorylated at critical residues,

i.e., two threonines and three serines (Jong *et al.*, 1993, Fleming *et al.*, 1995, Blaukat *et al.*, 1996, Blaukat *et al.*, 2001) and move to intracellular areas, becoming inaccessible and desensitized to agonists. The receptors are recycled to the cell surface by dephosphorylation and possibly by other events (Munoz *et al.*, 1993, Haasemann *et al.*, 1998, Lamb *et al.*, 2001). Internalization of the receptors seems essential for the resensitization process, since inhibition of internalization delays the dephosphorylation (Blaukat *et al.*, 1997).

Activation of NO synthesis by eNOS

When the BK-2R is activated by BK, eNOS is detached from the BK-2R- caveolin complex by dephosphorylation at Thr-497 and starts synthesizing NO within one second (Malinski *et al.*, 1992, Michel JB *et al.*, 1997, Marrero *et al.*, 1999). At the same time, the BK-2R signaling cascade progresses by activating PKC, which in turn causes eNOS to detach from Raf-1, MAPK and the protein kinase Akt. As a result, eNOS translocates to the cytosolic part of the cell (Michel T *et al.*, 1993, Prabhakar *et al.*, 1998, Harris *et al.*, 2001).

eNOS inactivation

In parallel with this movement of eNOS to the cytosol, the BK-2R signaling cascade activates the EGF receptor, which, in conjuction with activated PKC, starts the MAPK cascade by activation of Raf-1 (Adomeit *et al.*, 1999). Raf-1 next catalyzes the phosphorylation of MAPK through MEK (Blenis *et al.*, 1993, Fleming *et al.*, 1995, Seger *et al.*, 1995). Lastly, MAPK catalyzes the phosphorylation of eNOS and, parallel to this, the enzymatic activity of eNOS decreases to almost zero within five minutes of its initial activation (Bernier *et al.*, 2000). It is conceivable that the eNOS phosphorylation is the cause of the diminution of the eNOS signaling. A negative feedback mechanism following the eNOS activity has also been proposed by Fleming *et al.* (1999), who showed that tyrosine phosphorylation of MAPK also attenuates the intracellular increase of Ca^{2+} and that this inhibits the BK-2R signaling cascade and NO production.

The JAK/STAT pathway

In parallel with the events of eNOS activation, BK-2R also mobilizes other intracellular cascades, such as activation of the JAK/STAT pathway in the ECs. In resting ECs, BK-2R is attached to inactive tyk2, after which receptor dimerization induces tyk2 autophosphorylation followed by tyrosine phosphorylation of BK-2R. BK-2R phosphorylation produces a docking site for STAT3 (a transcription factor), which is subsequently tyrosine phosphorylated by tyk2. In parallel with these events, the activation of MAPK catalyzes the serine phosphorylation of STAT3, thereby maximizing the transcriptional activity of STAT3s. As a result of the BK-2R-initiated phosphorylation steps, STAT3 is translocated to the cell nucleus (Ju *et al.*, 2000).

NF-κB activation

BK-2Rs are also known to activate the nuclear factor-κB (NF-κB) pathway. BK-2R stimulation actuates the Gαq, Gβ1γ1, and GTPase RhoA-dependent signaling pathway that proceeds through phosphoinositide 3 kinase (PI3K), Akt, and IκB kinase

2 (IKK2), finally activating NF-κB (Pan ZK *et al.*, 1998 and 1999, Xie P *et al.*, 2000). NF-κB, in turn, activates the production of cytokines, for example interleukin-1β (Pan ZK *et al.*, 1996).

EDHF and its contribution to the vasodilatation produced by the BK-2R

BK-2R stimulation produces three vasodilatory agents, NO, prostacyclin and the so-called endothelium-derived hyperpolarizing factor (EDHF). The identity of the EDHF is not clearly defined and the term may represent a category of various compounds with the common denominator that they all cause hyperpolarization of vascular SMCs, thereby inducing blood vessel dilation. The many suggestions for the identity of EDHF may imply that different substances act in different tissues or animals (Cohen RA *et al.*, 1995, Fulton D *et al.*, 1997, Quilley *et al.*, 1997, Campbell WB *et al.*, 1999, Brandes *et al.*, 2000). The most probable candidate molecule for EDHF in the coronary, cerebral and renal arteries is epoxyeicosatrienoic acid (EET) (Campbell WB *et al.*, 1999, Halcox *et al.*, 2001, Pratt *et al.*, 2001, Rastaldo *et al.*, 2001). EET, the only cytochrome P450 metabolite of arachidonic acid in ECs, is the product of a P450 subtype, CYP 2C (Rosolowsky *et al.*, 1996, Fleming *et al.*, 2001). EETs can stimulate calcium-activated potassium channels in SMCs, causing hyperpolarization, relaxation, and vasodilatation (Campbell WB *et al.*, 1999).

EDHF is also responsible for a significant part of the vasodilatation induced by BK-2R. The contribution of EDHF to vasodilatation depends on the study system, probably reflecting differences in different vessels and varying from 10% to 50%. EDHF has been shown to be very important, particularly in the resistance vessels (Quilley *et al.*, 1997, Thollon *et al.*, 1999, Brandes *et al.*, 2000, Kamei *et al.*, 2000, Zhang DX *et al.*, 2001). It has been suggested to be the dominant mediator of BK-induced vasodilatation in the human forearm and coronary arteries, and possibly also in the renal arteries, underlining its importance in cardiovascular physiology (Miura *et al.*, 1999, Honing *et al.*, 2000, Bagate *et al.*, 2001b, Paolocci *et al.*, 2001). In addition to these findings, the study by Node *et al.* (1998), showing that EDHF is an important part of the BK-induced cardioprotection in acute MI in dogs, raises the question of the role of EDHF in human cardiovascular diseases. Lately, it has even been proposed that in some disease states, such as hypertension, BK-induced vasodilatation is mostly mediated by EDHF and not by NO. However, in diabetes, the vasodilating effects are mediated by NO (Taddei *et al.*, 1999, Wigg *et al.*, 2001), suggesting that different pathological processes influence the intracellular signaling pathways of BK-2R differently.

It was recently shown that in eNOS knockout mice, EDHF is able to completely replace NO in the vasodilatory effect of BK and that during inhibition of eNOS and prostaglandin synthesis, BK still induces vasodilatation through EDHF (Brandes *et al.*, 2000). Interestingly, increased production of NO has been shown to directly inhibit EDHF (Kessler *et al.*, 1999, Nishigawa *et al.*, 2000). Thus, it is likely that, under physiological conditions, NO inhibits EDHFs, but when eNOS is downregulated, EDHF may partially replace NO. Similar results have previously been shown for the other mediator of BK-2R, the prostaglandins, i.e. that prostaglandins can substitute NO for BK-2R-induced vasodilatation in the coronary circulation (Puybasset *et al.*, 1996).

Smooth muscle cells

Several recent findings have shown differences between the BK-2R-mediated signaling system in SMC and in other myocardial cell types. In SMC, the BK-2Rs are mainly located at the plasma membrane and not in the plasmalemmal caveolae , and stimulation of the BK-2R leads to a receptor translocation to plasmalemmal caveolae with subsequent internalization (Munoz *et al.*, 1993). As described previously, in ECs, and myocytes, and possibly in fibroblasts, activation of BK-2R seems to decrease the level of fibrosis and hypertrophy in the myocardium (McAllister *et al.*, 1993, Matoba *et al.*, 1999, Rosenkranz *et al.*, 1999). In SMCs in contrast, BK-2R stimulation induces especially the ERK/Elk-1/AP-1 pathway, which leads to activation of c-fos expression through reactive oxygen species (ROS), and to increased TGF-β production by vascular SMCs. Subsequently, this leads to increased cell proliferation and induction of fibrosis through increased collagen production and inhibition of the tissue inhibitor of matrix metalloproteinases (TIMP) (El-Dahr *et al.*, 1996 and 1998, Naidu *et al.*, 1999, Velarde *et al.*, 1999, Douillet *et al.*, 2000, Greene *et al.*, 2000, Yau *et al.*, 2001).
It also seems evident that direct stimulation of BK-2R on the SMCs leads to vasoconstriction instead of vasodilatation (Dixon *et al.*, 1994a). Kamei *et al.* (2000) made similar findings in the aortic rings of guinea-pig and Marsault *et al.* (1997) the canine saphenous veins. In absence of the endothelium, BK caused vasoconstriction of vessels, whereas in the presence of an intact endothelium, low concentrations of BK induced vasodilatation, while high concentrations caused vasoconstriction. These results suggest that activation of BK-2Rs located on ECs and on SMCs may lead to different cellular responses.

These controversial results may make sense in the critical process of tissue repair. Thus, in normal situations, BK stimulates predominantly the endothelial BK receptors, since the SMCs are located inside the vessel wall beneath the ECs. However, when the endothelium is damaged, BK may directly stimulate the BK-2R signaling in SMCs, causing contraction and proliferation, which would minimize the damage and initiate repair of the injury.

2.3.2. BK-1R

The BK-1R were characterized pharmacologically by Regoli *et al.* (1977) in the 1970s and structural analysis revealed that they were coded by an mRNA distinct from the BK-2R (Webb *et al.*, 1994). The gene, subsequently cloned from human embryonic lung fibroblasts, was shown to belong to the 7-transmembrane domain G-protein-coupled receptor family. The cDNA clone encoded 1307bp for a 353-amino acid protein with a molecular mass of approximately 41kDa (Menke *et al.*, 1994). Although the predicted amino acid sequence showed only 36% identity with the BK-2R, the two BK receptors are the closest relatives within the GPCR family. It was concluded that the BK-1R gene exists as a single copy on chromosome 14, more specifically q32 between markers D14S265 and D14S267 (Bachvarov *et al.*, 1996, Chai *et al.*, 1996, Yang X *et al.*, 1996).

The BK-1R gene is expressed in most major tissues and cell types of the circulation, although in some cases in very small amounts: blood vessels (Schremmer-Danninger *et al.*, 1998), kidney, lung, heart (Ni *et al.*, 1998b), ECs (Drummond *et al.*, 1995a,

Wohlfart *et al.*, 1997), SMCs (Tropea *et al.*, 1993, Schneck *et al.*, 1994, Drummond *et al.*, 1995b), fibroblasts (Menke *et al.*, 1994), and kidney cells (Wang DZ *et al.*, 1996) but not in CMCs (Minshall *et al.*, 1995, Clerk *et al.*, 1996).

2.3.2.1. Physiological effects of the BK-1R

Two characteristics of the receptor have influenced BK-1R research. Firstly, the receptor was originally found in rabbit blood vessels, where it is expressed in a constitutive manner, and secondly, the expression was found to be induced by inflammatory signals. Therefore the initial studies focused on its role in the circulation, especially during inflammation. Most of the studies of BK-1R have been made with animal models, i.e., with rats (practically all the hypertension studies and many on myocardial physiology), dogs (myocardial physiology) and mice (all the gene-knockout studies).

In the circulation, stimulation of BK-1R has been shown to cause vasodilatation. This seems to occur either in vessels with constitutive BK-1R expression, as in the dog, or in vessels in which the BK-1R expression has been induced by inflammation, for example by lipopolysaccharide (LPS) (Audet *et al.,* 1997, Su *et al.*, 2000). In addition, BK-1R has been suggested to precondition the heart against ischemic events, to protect it from arrhythmias (Chahine *et al.*, 1993, Feng *et al.*, 1997), and to be involved in the pathogenesis of diabetes (Zuccollo *et al.*, 1996). In addition, the BK-1Rs have been suggested to be involved in renal functions affecting both natriuresis and glomerular filtration (Fenoy *et al.*, 1992, Lortie *et al.*, 1992). In inflammation, BK-1Rs also have a role in leucocyte recruitment and the initiation of inflammatory responses (Pesquero *et al.*, 2000, Araújo *et al.*, 2001), as well as in the physiology of pain (Pesquero *et al.*, 2000). Lastly, BK-1Rs have been implicated to be mitogenic in fibrotic tissue, and like BK-2Rs, if expressed and stimulated in ECs to oppose fibrosis (Agata *et al.*, 2000, Parenti *et al.*, 2001).

2.3.2.2. Regulation of BK-1R

Under normal physiological conditions, the expression of BK-1R is very low (Bhoola *et al.*, 1992), but, under various inflammatory conditions, it has been shown to be increased (Galizzi *et al.*, 1994, Marin-Castano *et al.*, 1998). This is in sharp contrast to BK-2R, which are constitutively expressed under physiological conditions, and only modestly upregulated by inflammatory stimuli (Minshall *et al.*, 1995, Yang CM *et al.*, 2001). BK-1Rs have also been shown to be constitutively expressed in cats and dogs (Lortie *et al.*, 1992, DeWitt *et al.*, 1994). In addition to differences in basal expression and inducibility, the two BK receptors also differ in their responses to ligands. The BK-2R is both desensitized and resensitized by ligand stimulation, whereas the BK-1R is not (Windischhofer *et al.*, 1997, Faussner *et al.*, 1998 and 1999, Blaukat *et al.*, 1999, Zhou *et al.*, 2000, Marceau *et al.*, 2001). Thus, BK-1Rs may have potential in chronic diseases. Indeed, it has been suggested that BK-2Rs are essential for the kinin signaling at the beginning of inflammation, but that they gradually disappear. In contrast, BK-1Rs may be of minor importance in the acute phase of an inflammatory process, but as the inflammatory stimulus become chronic, BK-1Rs may overtake the role of BK-2Rs and become the key molecules in the chronic kinin signaling (Marceau *et al.*, 1998, Naicker *et al.*, 1999).

The BK-1R gene and its regulatory elements

Several different signals can induce BK-1R gene expression. It seems evident that the gene is tightly regulated by a negative regulatory element at –682 to –604bp upstream of the transcription initiation site. In addition, the gene contains binding sites for the **AP-1** factor, PEA3, CAAT, Sp1, Pit-1a, Oct-1, CREB, **NF-κB**, and **cAMP**-response elements (Ni *et al.*, 1998a, Yang X *et al.*, 1998, Angers *et al.*, 2000). Of these previous elements, the AP-1 factor seems crucial for full enhancer activity (Yang X *et al.*, 1998). Ni *et al.* showed that the region spanning from –2582 to +34 contains the regulatory elements for LPS, IL-1β, TNF-α, NF-κB, and the cAMP-response elements. Mutagenesis of the NF-κB element was sufficient to abolish most of the effects obtained with LPS, IL-1β, and TNF-α (Ni *et al.*, 1998a).

BK-1R upregulation by different stimuli

Inflammatory stimuli. The most efficient stimulus shown so far for BK-1R expression is LPS, which increases the BK-1R level by at least 3-fold (Regoli *et al.*, 1981, Marceau *et al.*, 1999). Similar stimuli seem to be pre-existing infection and Calmette-Guerin (BCG) vaccination, of which at least the former is mediated by LPS (de Campos *et al.*, 1998, Siebeck *et al.*, 1998). Interestingly, LPS seems to upregulate CPM, one of the two enzymes mainly responsible for the production of BK-(1-8), the ligand for BK-1R, thereby increasing the amount of this ligand (Schremmer-Danninger *et al.*, 1998). The effect of LPS is mediated through different inflammatory cytokines, most notably IL-1β (Ni *et al.*, 1998a, Zhou *et al.*, 1998, Campos *et al.*, 1999, Tsukagoshi *et al.*, 1999). Other inflammatory molecules promoting BK-1R upregulation include TNF-α, IL-8, and IFN-γ (Campos *et al.*, 1998 and 1999, Prat *et al.*, 2000). The cellular mechanisms of TNF-α, and especially of IL-1β, for induction of the BK-1R have been argued about but seem to include release of other cytokines, activation of PKC, and protein tyrosine kinase pathways, coordinated with the activation of MAPK, possibly SAPK/JNK and p38 MAP kinase, and lastly of NF-κB (Larrivee *et al.*, 1998, Ni *et al.*, 1998b, Zhou *et al.*, 1998, Sardi *et al.*, 1999, Medeiros *et al.*, 2001). PKC can also induce BK-1R directly through the activation of MAPK and NF-kB, suggesting that other PKC activating factors may also increase BK-1R levels (Zhou *et al.*, 1998)
Heat stress has also been shown to trigger BK-1R expression in an NO-dependent manner through the MAPK and p38 pathways (Lagneux *et al.*, 1997, 2000 and 2001).

Stimuli of the KKK system. In addition to inflammatory stimuli, BK-1R expression may also be increased by other factors. Both BK receptors, when stimulated, can induce BK-1R. BK-2R acts through induction of IL-1β expression and BK-1R through stimulation of PKC and NF-κB (Whalley and Nwator, 1989, Naraba *et al.*, 1998 and 1999, Schanstra *et al.*, 1998, Phagoo *et al.*, 1999). BK-1R stimulation has been shown to act synergistically with IL-1β in the lung fibroblasts, so that when BK-1R is stimulated alone, it uses p38 (MAPK) and when it is stimulated in conjunction with IL-1β, the NF-κB pathway is stimulated (Phagoo *et al.*, 2001). The concentrations used by Phagoo *et al.* (100nmol/l) are supraphysiological, indicating that BK-1R/2R-stimulated BK-1R upregulation may best apply to situations in which the ligand is synthesized to high levels, as in inflammation, or when its degradation is

reduced, as in ACE/NEP inhibition (Schanstra *et al.*, 1999). Interestingly, Duka *et al.* (2001) recently showed that in the BK-2R knockout mice, the BK-1R is upregulated, substituting partially, at least hemodynamically, for the lack of BK-2R. They also suggested that experimental manipulations producing hypertension can upregulate BK-1Rs. The same group later showed that, under physiological conditions, Ang II did not affect BK-1R expression, but that in BK-2R-KO mice, Ang II infusion further induced the already strongly upregulated BK-1R (Kintsurashvili *et al.*, 2001).

Growth factors. Several growth factors, such as PDGF, epidermal growth factor (EGF) and oncostatin M, have been shown to induce BK-1R in aortic preparations and cells. Of these growth factors, at least EGF uses the MAPK second messenger pathway (Bouthillier *et al.*, 1987, Larrivee *et al.*, 1998).

Diseases. MI and ischemia-reperfusion have recently been shown to induce BK-1R expression in rat and rabbit hearts (Tschöpe *et al.*, 2000, Mazenot *et al.*, 2001), which raises important questions about the role of BK-1R in the pathophysiology of cardiovascular diseases. These investigators speculate that BK-1Rs may be involved in tissue repair.

Signals inhibiting BK-1R expression

Although several systems have been shown to induce BK-1R expression, little is known about the suppression of the gene. The presence of a negative regulatory element in the BK-1R gene has been suggested to keep the gene in total control under normal physiology (Yang X *et al.*, 1998). So far, only two inhibitory signals have been shown for BK-1R expression: glucocorticoids and p53. In adrenalectomized rats, i.e. in rats with low glucocorticoid levels, regardless of whether induced surgically or chemically, the BK-1R are upregulated. This effect is transmitted through COX-2 and NF-κB, and if these adrenalectomized animals are given glucocorticoids, the BK-1R is again downregulated to normal levels (Cabrini *et al.*, 2001). Although the exact mechanisms by which p53 suppresses the promoter activity of the BK-1R are presently unknown, it has been shown that suppression by p53 is not mediated by interaction with a putative p53-binding site, or by competition with the TATA-binding protein or with c-Jun, which functions in the inducibility of the gene (Yang X *et al.*, 2001).

2.3.2.3. The second messengers of BK-1R

After binding of ligands to the BK-1R, the G-proteins q and i_2 are activated, followed by PLC and IP_3 activation and increases in cGMP and intracellular Ca^{2+} (Austin *et al.*, 1997, Agata *et al.*, 2000, Prat *et al.*, 2000). As a result of BK-1R activation, COX 1 and 2 start producing PGE_2 and PGI_2 (Levesque *et al.*, 1995, Meini *et al.*, 1998, McLean *et al.*, 1999) and, in addition, eNOS is activated to produce NO (Drummond *et al.*, 1995 and 1995, Belichard *et al.*, 1996, Agata *et al.*, 2000, Prat *et al.*, 2000, Su *et al.*, 2000). The BK-1R signaling cascade induces vasodilatation in resistance arteries of the myocardium in dogs (Drummond *et al.*, 1995, Belichard *et al.*, 1996, Su *et al.*, 2000), and inhibits SMC growth via NO signaling (Dixon *et al.*, 1997, Agata *et al.*, 2000, Emanueli *et al.*, 2000), but induces growth in ECs via eNOS-mediated upregulation of FGF-2 (Parenti *et al.*, 2001).

In addition, activation of the MAPK pathway and of the PKC pathway both lead to activation of NF-κB (Levesque *et al.*, 1995, Schanstra *et al.*, 1998, Campos *et al.*, 1999, Christopher *et al.* 2001), with the production of cytokines (IL-1, IL6 & 8, TNF-α), growth factors (FGF) and collagen, leading to tissue growth and fibrosis (Tiffany *et al.*, 1989, Levesque *et al.*, 1995, Phagoo *et al.*, 1999, Ricupero *et al.*, 2000, Parenti *et al.*, 2001). As in the BK-2R system, the seemingly controversial inhibition and promotion of fibrosis by the same receptor probably reflect cell specific differences in intracellular signaling. Similarly, prostaglandins, NO produced by the activation of BK-1Rs in ECs inhibit SMC growth, whereas direct stimulation of BK-1R in SMCs may promote their growth.

3. Influence of kinins on diseases contributing to the pathogenesis of heart failure and on heart failure directly

3.1. Diabetes

The KKK system has been suggested to have a protective role in the heart and the kidneys during the progression of diabetes, mainly by affecting vasodilatation and the metabolism of glucose. However, the overall evidence for the role of the KKK system in diabetes is still questionable.

3.1.1. Effect of diabetes on the control of expression of kininogen-kallikrein-kinin components

It has recently been suggested that synthesis of the components of the KKK system is influenced by diabetes and insulin (Mayfield *et al.*, 1996). This suggestion is supported by several studies on kinin synthesis in both the plasma and the tissue compartments. Mayfield *et al*. (1995) first showed that streptozotocin-induced diabetes in rats (STZ rats) causes downregulation of the KKK system (mainly pKLK) and hypertension, a finding confirmed by others (Tschöpe *et al.*, 1999a & 1999b). The studies by Rotschild *et al.* (1996 and 1999) suggest that, in diabetes, a decrease in insulin impairs pKLK-HMW kininogen activation, causing a decrease in pKLK and BK levels, with secondary increases in plasma HMW kininogen and prokallikrein. In a similar experiment, insulin dose-dependently corrected both prokallikrein and kininogen levels in STZ rats. In parallel, the BK-2Rs can increase salt-excretion in experimental diabetes (Zuccollo *et al.*, 1996). Indeed, when STZ rats were treated with insulin, they showed glomerular hyperfiltration with an increase in renal KLK synthesis (Mayfield *et al.*, 1985, Jaffa *et al.*, 1992), an effect that was counteracted by the BK-2R antagonist or aprotinin (a KLK inhibitor) (Jaffa *et al.*, 1987, Harvey *et al.*, 1990).

In addition to pKLK, tKLK is also downregulated in both kidney, plasma and heart during experimental diabetes (Sharma *et al.*, 1996 and 1998, Tschöpe *et al.*, 1996, 1997 and 1999, Jaffa *et al.*, 1997), resulting in increased levels of LMW kininogen in plasma (Uehara *et al.*, 1988). Interestingly, the downregulation of tKLK can be restored to normal levels by insulin and possibly by exercise (Chan *et al.*, 1993, Koh *et al.*, 1988).

A disagreeing study was recently published showing increased levels of BK and BK-(1-7) in the hearts of diabetic Sprague-Dawley rats as compared with their controls, further suggesting that kinin degradation is upregulated in the heart (Campbell DJ *et al.*, 1999b). There are also suggestions that kininogen, the substrate of KLK, may be decreased in the heart during diabetes (Sharma *et al.*, 1999). Thus, the downregulation of the KKK system in diabetes may be a maladaptive response, which participates in the pathogenesis of LVH in STZ rats (Sharma *et al.*, 1998).

3.1.2. The kininogen-kallikrein-kinin system in the pathophysiology of diabetes: its relation to insulin and glucose metabolism

In diabetes, the major pathophysiological change is the decrease in plasma insulin levels, which causes an increase in blood glucose, that, affects many different tissues, especially the endothelium. BK, which is increased by ACEi, has been shown to decrease blood glucose and, therefore, is a potential target for treatment in diabetes (Jaffa *et al.*, 1986, Rett *et al.*, 1986, Rosenthal *et al.*, 1997). BK may affect diabetes by several different mechanisms: 1) by increasing glucose uptake, 2) by improving the use of glucose, and 3) by improving glucose tolerance in both patients and severely insulin-resistant rats (Wicklmayr *et al.*, 1979, Hartl *et al.*, 1990, Henriksen *et al.*, 1998, Damas *et al.*, 1999). The most important mechanism seems to be the increased translocation of the glucose transporter protein 4 (GLUT4) (Kishi *et al.*, 1998). The transport of glucose was shown to be increased 3-fold in the presence of ACEi or BK, and the effect was abolished by HOE-140, a BK-2R blocker (Rett *et al.*, 1997). BK has been shown to affect both insulin-dependent and insulin-independent uptake of glucose by skeletal muscle cells (Kishi *et al.*, 1998, Kudoh *et al.*, 2000a). Inside the muscle cells, the effect is mediated through G-protein (type q), PLC, tyrosine kinase and an increase in intracellular Ca^{2+} (Kishi *et al.*, 1998, Kudoh *et al.*, 2000b).

In addition to affecting glucose metabolism, BK also interacts with insulin directly. BK has been shown to increase the release of insulin after a glucose load through BK-2R in the β-cells of the Langerhans islets (Yang C *et al.*, 1997, Damas *et al.*, 1999). It has also been shown that BK, through BK-2R, increases insulin sensitivity in normal and obese rats and in human non-insulin-dependent diabetics (Rett *et al.*, 1986, Jauch *et al.*, 1987, Uehara *et al.*, 1994, Kohlman *et al.*, 1995, Arbin *et al.*, 2001), an effect that could be further enhanced by ACEi (Uehara *et al.*, 1994, Arbin *et al.*, 2001). The observed effect of BK may be the result of enhanced intracellular signaling cascades of insulin (Miyata *et al.*, 1998, Motoshima *et al.*, 2000).

Exercise has been shown to decrease the levels of glucose and to increase the levels of BK in plasma by increasing tKLK activity (Koh *et al.*, 1988, Taguchi *et al.*, 2000). Indeed, it was shown that BK-2R antagonists (HOE-140) abrogated the exercise-induced glucose transport in rats, suggesting that the effect was mediated by BK-2Rs (Kishi *et al.*, 1998, Taguchi *et al.*, 2000). The interplay between BK-2Rs and insulin seems to work both ways, since insulin, by increasing the production of IP3 and decreasing BK-2R-induced tyrosine phosphorylation, also affects BK-2R signaling (Haring *et al.*, 1996, Kudoh *et al.*, 2000a). Interestingly, ACEi has been shown to reduce the oxidative stress in hyperglycaemic conditions by activating the BK/BK-2R signaling pathway (Rumble *et al.*, 1996, Mikrut *et al.*, 2001).

3.1.3. Bradykinin and vasodilatation in diabetes

In diabetes, the high level of glucose impairs the endothelial function (Rosenkranz *et al.*, 1999). Since BK is one of the most potential peptides with capability to stimulate NO secretion in the vasculature, it is important to know whether the BK-mediated endothelial-dependent vasodilating effect is impaired in diabetes. Indeed, it has been shown that, in diabetes, BK-induced vasodilatation is impaired in the coronary and mesenteric arteries, and especially in the small arteries, in addition to the aorta and the kidneys (Quilley *et al.*, 1992, Vora *et al.*, 1997, Costa e Forti *et al.*, 1998, Shen *et al.*, 1999, Zhao *et al.*, 1999, Ding *et al.,* 2000, Vallejo *et al.*, 2000, Rizzoni *et al.*, 2001a and 2001b).
There are several potential mechanisms by which vasodilatation may be impaired in diabetes. First, the amount of eNOS protein and its activity have been shown to be downregulated in diabetes (Zhao *et al.*, 1999), whereas both insulin and BK-2R stimulation are capable of attenuating the inhibitory effects of glucose on eNOS expression (Ding *et al.*, 2000, Bachetti *et al.*, 2001). Secondly, in addition to inhibition of eNOS expression, high glucose may inhibit the eNOS-mediated vasodilatation by scavenging NO. In addition, glucose may also induce a decrease in cellular Ca^{2+} by increasing the levels of hydroxyl radicals. Both these mechanisms interfere with BK-mediated vasodilatation (Pieper *et al.*, 1997, Brodsky *et al.*, 2001, Goligorsky *et al.*, 2001). Thirdly, in addition to a reduced level of NO, the BK-induced EDHF-mediated vasodilatation is also reduced in diabetes (Wigg *et al.*, 2001). Interestingly, in co-cultures of ECs and CMCs, hyperglycemia, which causes endothelial dysfunction, including a reduction in the level of NO, also impairs the BK-mediated antihypertrophic response in CMCs (Rosenkranz *et al.*, 1999).

Overall, diabetes seems to downregulate the KKK system, thereby causing impairment in both vasodilatation and glucose metabolism. In addition, this may impair salt handling in the kidney and induce endothelial dysfunction and LVH in the heart.

3.2. Hypertension

To date, the pathology of essential hypertension is not clear, but most likely both impaired renal and vascular physiology, especially endothelial dysfunction, play major roles. In both areas, the KKK system seems to be involved.

Most of the data concerning the pathophysiology of essential hypertension, including the kinin system, has originated from studies with experimental animals, i.e. rats. The most important rat model is the spontaneously hypertensive rat (SHR); others include deoxycortisone-acetate (DOCA)-salt, and renal-clip-, aortic-banding- and salt-loading- induced models of hypertension.

3.2.1. Regulation of the kininogen-kallikrein-kinin system in hypertension

In the SHR model, both plasma and cardiac kininogen levels, as well as tissue KLK levels, have been shown to be reduced (Sharma *et al.*, 1998 and 1999). In contrast, the kininase levels in plasma are not affected (Dendorfer *et al.*, 2001b). However, in the kidneys of SHRs the kinin concentrations are increased (Campbell *et al.*, 1995). In

addition, Stier *et al.* (1992) showed that stroke-prone SHR with established hypertension has an enhanced vasodilatory response to BK in the kidney, suggesting upregulation of either renal BK-2Rs or BK-1Rs. Interestingly, in spite of increased formation of renal kinins and increased expression of renal kinin receptors, hypertension is a prominent feature of the SHR model.
A non-peptide BK agonist has been shown to blunt hypertension in the young SHR, suggesting a major role for the kinins in this model (Majima *et al.*, 2000). Also, the BK-2R blocker, HOE-140, has been shown to increase blood pressure in the WKY (the control strain of SHR), but not in the SHR, suggesting that, without inhibition of their degradation, the level of kinins would be too low to have a major impact on the blood pressure (Emanueli *et al.*, 1997b). A reasonable explanation for these results may be that the KKK system regulation differs between the tissues, i.e. hypertension upregulates kinin and the kinin receptor levels in the kidney, while the formation of kinins in the myocardial and peripheral tissues is downregulated, increasing the vascular resistance. This would suggest that in the SHR model the KKK system may affect hypertension through effects on the peripheral vasculature (or plasma) and cardiac tissue, while in the renal tissue they would be ineffective.

In some other studied models of hypertension, the overall kinin-generating activity was found to be upregulated, and the kinin concentrations could be further increased by ACEi (Chao C *et al.*, 1996, Nolly *et al.*, 1997, Miatello *et al.*, 1998). Regarding the renal kinins overall, it has been shown that KLK expression is genetically determined in that salt-resistant kidneys have a higher expression of KLK than salt sensitive ones (Churchill *et al.*, 1995), and conversely, low-salt conditions have been shown to downregulate pKLK levels (Hilgenfeldt *et al.*, 1998). In addition, the kinin concentrations are increased in the kidneys of several hypertensive rat models (Jin L *et al.,* 1999, Mackie *et al.*, 2001).
Therefore, increased KLK synthesis does seem to be an important measure used by the kidneys for protection against hypertension, whereas disturbances in this protective system may result in increased blood pressure. Since the results concerning the levels of the KKK system components in humans are controversial, further studies are needed (Hughes *et al.*, 1988, Sanchez *et al.*, 1996) to verify the role of the KKK system in human hypertension. On the basis of these findings it can be concluded that, in SHRs, the kinin system is activated in the kidneys but downregulated in other tissues, resulting in an ineffective measure for opposing the development of hypertension.

3.2.2. The systemic vascular effects of the kininogen-kallikrein-kinin system

One of the major pathological changes in essential hypertension is the induction of endothelial dysfunction, including a general deficiency in vasodilatation similar to that produced by lack of BK (Panza *et al.*, 1995). Indeed, ACEi have been shown to restore both the function of ECs and the capacity for vasodilatation in SHR through a BK-dependent mechanism (Kähönen *et al.*, 1995, Bennett *et al.*, 1996). Similarly, it has been shown that ACEi increases the response to exogenous BK infusion both acutely and chronically (Taddei *et al.*, 1998).

It has been suggested that endogenous BK and ACEi, acting through endogenous BK, affect only the acute, but not the chronic blood pressure homeostasis (Rhaleb *et al.*, 1999). Consequently, acute infusion of ACEi or AT_1 antagonist into humans or

animals, the latter activating the AT-2/BK/BK-2R/NO pathway, causes vasodilatation and hypotension via the BK-2R (Carbonell *et al.*, 1988, Holte *et al.*, 1996, Gainer *et al.*, 1998, Gohlke *et al.*, 1998).
However, kinins have also been shown to have an effect on the chronic regulation of blood pressure. Both a five-day semi-acute and a chronic ACEi treatment (several weeks) showed a kinin-dependent reduction in blood pressure in Wistar and Wistar Kyoto (WKY) rats (Emanueli *et al.*, 1997a , Bagate *et al.*, 2001) and Majima *et al.* (2000) have also shown that chronic infusion of a non-peptide BK analog in young SHRs (6 to 8 weeks) blunted the development of hypertension.
Furthermore, chronic co-infusion of BK and Ang II into rats attenuates the Ang II-induced effects of peripheral vascular resistance, and, at the same time, BK also increases the cardiac stroke volume and the cardiac output (Pasquie *et al.*, 1999).
Thus, it seems evident that the kinin system is able to affect blood pressure both acutely and chronically, one of the mechanisms being attenuation of the effects of the RAS system.

The evidence from the transgenic mouse models

In the light of experiments with the BK-2R knockout mouse (BK-2R-KO), which develops hypertension and HF under basal conditions, it has also been suggested that BK-2Rs are essential for the maintenance of normal blood pressure (Emanueli *et al.*, 1999).

For a start it was shown that chronic infusion of captopril caused a greater decrease in blood pressure in BK-2R-KO mice than in controls, indicating that an intact kinin system directly counteracts the vasoconstrictor effect of Ang II (Emanueli *et al.*, 1997a). This observation was confirmed by Cervenka *et al.* (2001), who showed that Ang II-infusion had more extensive hypertensive effects in BK-2R-KO mice than in controls. Furthermore, acute inhibition of nitric oxide synthase augmented the blood pressure of the control mice to a level similar to that in BK-2R-KOs, suggesting that the enhanced susceptibility of BK-2R-KO mice to Ang II-induced hypertension depended on the impaired ability of endogenous kinins to release NO. In addition, both hypertension and LVH in BK-2R-KOs could be inhibited by AT_1 antagonists (Emanueli *et al.*, 1999, Madeddu *et al.*, 2000).

Interestingly, Duka *et al.* (2001) further confirmed the hypertensive phenotype of BK-2R-KOs in a strain with a mixed c57Bl mouse background. In addition, they also showed that BK-1Rs are upregulated in BK-2R-KO mice, assuming some of the hemodynamic properties of BK-2Rs. These data suggest that future studies with BK-2R-KO mice should also take into account the possible substitutional effects mediated by BK-1Rs in the absence of BK-2Rs.

The role of BK-2R in hypertension has been further verified by showing that overexpression of human BK-2R in mice and of tKLK in rats induces hypotension in these animal models (Song *et al.*, 1996, Wang DZ *et al.*, 1997, Zhang JJ *et al.*, 1999). In parallel, in rats overexpressing the human KLK gene the isoproterenol-mediated hypertension and LVH were reduced, and both effects were blunted with HOE-140 (Silva *et al.*, 2000).

In contrast to accumulating evidence of the involvement of BK-2R in hypertension and HF, Carreteros group (Madeddu *et al.*, 1999, Rhaleb *et al.*, 1999, Yang XP *et al.*, 2001) were unable to show either hypertension or cardiac abnormalities in BK-2R-KOs. This observation has also been supported by Milia *et al*. (2001), who were unable to show any differences in basal or salt-challenged blood pressure in BK-2R-KO mice. However, the latter group also suggests that additional systems, such as the upregulation of tKLK, may compensate for the loss of BK-2Rs in their BK-2R-KO strain.

In spite of these contradictory observations, it may be concluded that BK-2Rs, as part of the KKK system, participate in the endogenous cardiovascular regulatory system that prevents the RAS from inducing hypertension under normal physiological conditions. Furthermore, the most important second messenger in BK-mediated vasodilatation seems to be NO.

3.2.3. The renal kininogen-kallikrein-kinin system in hypertension

Hypertension can arise from defects in salt handling or in renal blood flow, and early findings showed that kinins stimulate water and electrolyte excretion by increasing renal blood flow (Webster and Gilmore, 1964, Gill *et al.*, 1965). The increased flow is caused by decreased vascular resistance and does not affect the glomerular filtration rate or the absolute proximal reabsorption. However, BK can induce a marked increase in fluid delivery to the distal nephron (Stein *et al.*, 1972), and natriuresis seems to depend on inhibition of sodium reabsorption distally (Roman *et al.*, 1988, Tornel *et al.*, 2000). Conversely, low salt conditions have been shown to downregulate pKLK excretion, which decreases the formation of kinins (Hilgenfeldt *et al.*, 1998).

Results of direct manipulations on the kininogen-kallikrein-kinin system.

Even endogenous kinins can have a powerful effect on salt-handling, and the effect can be further enhanced by ACEi (Fenoy *et al.*, 1992). Tornel *et al.* (2000) further showed that HOE-140, a BK-2R antagonist, can induce the retention of salt and water and thus cause strong long-term hypertension.
The role of BK in salt-induced hypertension has been extensively studied. Experiments performed in 1) kininogen-deficient Brown-Norway-Katholiek rats (BNK), 2) rats inbred for low KLK excretion, 3) BK-2R-KO mice, 4) Dahl salt-resistant rats with BK-2R blockade and 5) normal rats treated with anti-KLK antibody consistently showed that a salt challenge, on top of an impaired KKK system, makes these animals hypertensive. Some of them also show a marked retention of Na^+ and H_2O (Majima *et al.*, 1993, Saitoh *et al.*, 1995, Alfie *et al.*, 1996 and 1997, Madeddu *et al.*, 1996, Mukai *et al.*, 1998, Cervenka *et al.*, 1999).

In addition, the BK-2R-KO mouse has decreased renal blood flow, and develops salt-sensitive experimental hypertension much faster than the control mice, and the hypertension is more severe (Cervenka *et al.*, 2001, Duka *et al.*, 2001). It has also been shown that BK-2R normally opposes the antidiuretic effect of arginine vasopressin (Alfie *et al.*, 1999) and that transgenic mice overexpressing BK-2R and rats overexpressing tKLK have enhanced renal function and are hypotensive (Chao J *et al.*, 1997, Wang D *et al.*, 2000).

Kinins and the RAS in renal physiology

One of the key mechanisms of kinins in opposing hypertension by affecting renal physiology seems to be, as in vascular physiology, counteraction of the RAS. Indeed, it has been shown that when BK is infused with Ang II into rats, it attenuates the Ang II-induced effects on renal vascular resistance and renal blood flow (Pasquie *et al.*, 1999). Conversely, the lack of a fully functional KKK system in low-KLK rats allows Ang II to have an unbalanced action on renal function leading to increased glomerular hydrostatic pressure and reduced excretion of Na^+ (Madeddu *et al.*, 2001b).

AT_2, on the other hand, has effects opposite to AT_1, such as induction of salt excretion and renal vasodilatation. These effects are enhanced when AT_1 is blocked and have been shown to use the signal transduction pathway that leads to increased BK concentration and subsequent stimulation of BK-2R (Munoz-Garcia *et al.*, 1995, Siragy *et al.*, 1997, Carey *et al.*, 2000, Moore *et al.*, 2001). In renal wrap hypertension, AT_2R blockade caused a further increase in the already high blood pressure and was shown to directly decrease BK and NO concentrations in the renal interstitium (Siragy *et al.*, 1999).

In conclusion, activation of the KKK system causes natriuresis, and, on the other hand the KKK system itself is activated in the kidneys with high- and inactivated with low-salt conditions, its absence leading to salt sensitivity of the blood pressure regulation. On the other hand, the KKK system opposes hypertension by counteracting the RAS in the kidney. Overall it can be concluded that the KKK system affects the pathogenesis of hypertension through both renal and vascular physiology, the key mechanisms for this including the opposition of the RAS.

3.3. The role of kinins in ischemic and ischemia-reperfusion injuries

Kinins, in addition to acting against diabetes and hypertension, have been shown to exert cardioprotective effects in ischemia-reperfusion injuries. These effects are related to cardioprotection both during and after ischemia-reperfusion injury and to a cardioprotective phenomenon called preconditioning (PC). Since the fundamental mechanisms behind each phenomenon are still unclear, it may be that the effects are either variations on the same theme, i.e. have the same mechanisms but with different emphases, or two totally different entities. As the intracellular cascades of each are revealed, it will be possible to make a final judgment about this.

3.3.1. Ischemia-reperfusion injury

During the classical ischemia-reperfusion insult, a stop in blood flow results in impairment of the oxygen and the glucose supply and accumulation of the various products of energy metabolism. Even during the first minutes, the lack of oxygen affects the parenchyma, especially the CMCs, the most vulnerable cells in the heart. When reperfusion is started, it does not fully correct the situation, as it triggers inflammatory processes, including infiltration of white blood cells and production of cytokines and other inflammatory molecules. In addition to injury and death of myocytes, these events result in arrhythmias, impaired contractile function, and later,

in myocardial remodelling processes (Antman and Braunwald, 2001). Kinins can potentially affect the myocardial metabolism in ischemic situations in two ways, either through activation of ECs causing vasodilatation or through direct protective effects on myocytes. These effects are difficult to separate since the main effector molecule of the EC is NO but the CMCs can also produce NO in response to BK and this has been shown to directly protect the myocytes from ischemia (Wall *et al.*, 1996, Matoba *et al.*, 1999).

Kinin-mediated vasodilatation, and endothelial dysfunction

Kinin-mediated vasodilatation is, under normal circumstances, mainly mediated by endothelium-derived NO, and during ischemia by both NO and EDHF (Node *et al.*, 1998). The importance of BK in the physiology of blood flow was underlined by the recent finding that BK-mediated vasodilatation can directly influence the myocardial contractile function (Node *et al.*, 1997).

In patients with CHD, it has been shown, that endothelial dysfunction takes place in both the coronary and the peripheral arteries, which means impaired vasodilatation in response for example, to kinins. The impairment includes both endothelium-dependent and endothelium-independent vasodilatation, and more specifically affects the atherosclerotic sites, vasodilatation being preserved at spastic sites (Kuga *et al.*, 1995, Benacerraf *et al.*, 1999). ACEi treatment can restore the endothelium-mediated part of the vasodilatation both at the periphery and in the coronary arteries, and in patients with endothelial dysfunction can also repair the abnormal flow-mediated epicardial vasomotion, partly via BK (Prasad *et al.*, 1999). This suggests that in CHD the baseline kinin concentrations or activity of the BK-2Rs for protection against endothelial dysfunction are not maximal.

The kinin levels

It has been shown that, during a cardiopulmonary bypass operation, MI and, in inflammation, the kinin formation system is upregulated (Pang *et al.*, 1979, Chao J *et al.*, 1988, Saatvedt *et al.*, 1995, Hoffmeister *et al.,* 1998 and 1998, Wendel *et al.*, 1999). In these situations, in addition, the decrease in myocardial pH has been shown to impair BK degradation (Ahmad *et al.*, 1996). Consequently, in the human cardiopulmonary bypass operation and in the rat MI model the BK levels have been shown to be increased in cardiac tissue, in the urine and in the circulation (Matsuki *et al.*, 1987, Campbell *et al.*, 1995, Gonzalez *et al.*, 1996, Campbell *et al.*, 1997, Blais *et al.*, 2001, Campbell DJ *et al.*, 2001).
However, after infarction and reperfusion, myocardial kinin degradation has been shown to increase, and this probably reduces the kinin concentrations to the normal levels or even below them (Raut *et al.*, 1999). Inhibition of the degradative enzymes after the acute situation should therefore be (and has been shown to be) effective in promoting recovery of the heart. In conclusion, during an acute ischemic incident, kinins are increased, while afterwards they are decreased.

The effects of kinins on myocardial physiology during ischemia and reperfusion

The positive effects of kinins on recovery from MI were found by infusing BK into animals with MI. This protected the myocardium from ventricular fibrillations by

promoting electrical stability, reducing lactate and creatinine kinase (CK) activities, improving cardiodynamic and metabolic parameters and improving recovery from the insult, regardless of whether the infusion was given before the ischemia or during reperfusion (Linz *et al.*, 1986, Schölkens *et al.*, 1988, Tobe *et al.*, 1991, Zhu *et al.*, 1995, Abbas *et al.*, 1999).

In parallel with the effects of exogenous BK infusion, studies have been made of how enhancing the kinin system by increasing kinins affects recovery of myocardium from ischemia-reperfusion injuries. It has been shown that, during ischemia, ACEi increases myocardial NO levels through increased BK (Kitakaze *et al.*, 1998). Schölkens´ group showed already in 1988 that ACEi ramipril improved cardiac performance, increased coronary flow, reduced arrhythmias and showed positive effects on the biochemical markers of ischemia in ischemic hearts and that HOE-140 blocked these effects (Linz *et al.*, 1987, Schölkens *et al.*, 1988). In addition to these, and very importantly, it was shown that ACEi reduces MI size and that the effect is blocked by HOE-140 (Hartman *et al.*, 1993, Rump *et al.*, 1993). Interestingly, in the absence of ACEi, HOE-140 also increased infarct size, although not significantly (Hartman *et al.*, 1993). These results were confirmed by others (Fleetwood *et al.*, 1991, Massoudy *et al.*, 1994, Cargnoni *et al.*, 2001, Whang *et al.*, 2001) and similar effects have since been shown with the AT_1 antagonist and APP inhibitor (Ersahin *et al.*, 1999, Zhu *et al.*, 1999).

Lastly, in both the mouse KLK gene delivery model and in the transgenic rat model overexpressing tKLK, the myocardial BK is increased and causes attenuation of MI by reducing the size of infarct, the incidence of arrhythmias, and apoptotic cell death in the ischemic area (Pinto *et al.*, 2000, Yoshida *et al.*, 2000).

If increased kinin concentrations offer cardioprotection, then what about the effects of endogenous kinins? Indeed, it has been shown that the BK-2R antagonist (CP-0127) impairs recovery from acute coronary ischemia by increasing coronary vascular resistance and depressing myocardial function (Neuhof *et al.*, 1997). The antagonist has also been shown to increase the incidence of sustained ventricular fibrillations from 42% to 100%, and interestingly ACEi could further reduce the “basal level” (42%) of arrhythmias by 70%, provided that BK-2R was not blocked (Shimada *et al.*, 1996). Lastly the BK-2R blockade seems to increase infarct size (Hartman *et al.*, 1993). Conversely, these results mean that in ischemia-reperfusion injuries, the endogenous levels of kinins are already protective for the heart, but ACE inhibition or other measures that increase the kinin concentrations can increase their positive effects still further.

3.3.2. Preconditioning

Preconditioning (PC) was first described by Murry *et al*. (1986), who showed in a dog model that the size of a MI subjected to coronary artery occlusion for 40 minutes was greatly reduced if the dog was first subjected to 4 brief periods of ischemia (5 minutes) followed by reperfusion (5 minutes). This effect is achieved within minutes of initiation and is generally called classical or ischemic PC to distinguish it from the other type of PC, which is paradoxically called late PC, or the second window of protection. Late PC involves a cardioprotective response that becomes effective only 5 to 6 hours after a stimulus and has a less potent protective effect. However, the

cardioprotective effect of late PC lasts much longer, i.e. up to several days after initiation (Yellon & Baxter, 1995, Bolli *et al.*, 1998).

PC has been shown to take place in all major experimental animals and there is strong circumstancial evidence that this phenomenon also takes place in the human heart (Speechly-Dick *et al.*, 1995, Ikonomidis *et al.*, 1997). Interestingly, one of the key signals inducing PC is BK, others being adenosine, opioids and free radicals (Liu GS *et al.*, 1991, Vegh *et al.*, 1991, Schultz *et al.*, 1995, Baines *et al.*, 1997).

Classical PC

The first study showing that BK induces PC was published by Vegh *et al*. (1991), who showed that infusion of BK into a small branch of the left anterior descending coronary artery in dogs prior to and during occlusion dramatically decreased ventricular fibrillations and tachycardias as compared with saline infused control dogs.

The effect of ischemic PC on kinin concentrations. Ischemia was first shown to enhance kinin formation (Parratt *et al.*, 1997), indirectly implying increased myocardial kinins. Recently, this has also been shown in pigs and cats, in which the concentration of endogenous BK increased 200% after 3 minutes of ischemic PC and remained high if the PC lasted for up to 10 minutes (Schultz *et al.*, 1998). The BK-2R antagonist, HOE-140, abolished both the increase in BK and the PC effect, implying a role for BK-2R in the process. In addition, interstitial BK concentrations in the heart rose 10-fold during myocardial ischemia in PC cats compared with non-PC cats (Pan HL *et al.*, 2000). In a third study in dogs, ACEi increased the BK concentration 100% before and during subsequent regional ischemia, resulting in reduced mortality, infarct size, incidence of arrhythmias and increased overall contractility (Hosoya *et al.*, 2000).

Ischemic PC and kinins. It has been shown both *in situ* and *in vivo* that ischemic PC can greatly reduce the infarct size, decrease arrhythmias, and at least partially correct the endothelial dysfunction that develops after ischemia through a BK-2R mediated mechanism (Goto *et al.*, 1995, Giannella *et al.*, 1997, Kaszala *et al.*, 1997, Sato M *et al.*, 2000).
In addition, kininogen-deficient rats (Brown Katholiek Norway rats) and BK-2R-KO-mice have been shown to have impaired PC responses, showing that an intact system for kinin formation and intact kinin receptors are needed for the BK-mediated cardioprotective effect of ischemic PC (Yang XP *et al.*, 1997).
In contrast, in isolated rat hearts the group of Ytrehus was unable to show any role for infused BK in ischemic PC (Bugge *et al.*, 1996, Starkopf *et al.*, 1997).

Induction of PC. Since an increase in kinins can induce PC, it might be even more beneficial to increase the endogenous kinin concentration further in order to induce the PC effect. This can be achieved either by infusing BK itself or by using ACEis to increase the concentration of endogenous kinins by causing decreased kinin degradation. Liu *et al.* (1996) showed that preischemic ACE inhibition through the KKK system (an effect inhibitable by HOE-140, a BK-2R blocker) reduced myocardial infarct size and arrhythmias in rats and the results were confirmed in rats (Tanonaka *et al.*, 1996, Yang X *et al.i,* 1996b) and rabbits (Miki *et al.*, 1996). On the

other hand, losartan, an AT_1 antagonist, was also shown to reduce myocardial ischemia/reperfusion injury through BK-2R and prostaglandin-dependent mechanisms (Jalowy *et al.*, 1998, Zhu *et al.*, 1999, Sato *et al.*, 2000).

Second Messengers. The key step in PC-mediated intracellular signaling has been shown to be the activation of PKC-ε followed and/or paralleled by tyrosine kinase activation (Brew *et al.*, 1995, Goto *et al.*, 1995). This leads to activation of the p38 MAPK cascade and in some species also the JNK cascade (Cohen MV *et al.*, 2000), i.e. signaling effects that are not specific for BK. In addition, Cohen *et al.* (2001) showed that free radicals are also needed for BK-mediated PC. The downstream signaling pathway is still unclear, except for the last step, i.e. the opening of mitochondrial ATP-sensitive K^+channels (Kita *et al.*, 2000, Ren *et al.*, 2001).

In addition to PKC, NO and EDHF have also been proposed to play a role in PC against arrhythmias (Parratt *et al.*, 1994, Node *et al.*, 1998, Kis *et al.*, 1999). Yoshida *et al.* (1999) have shown that NO causes PKC translocation and improved recovery of the function of the heart. Recently, it was shown that coronary infusion of BK into isolated rabbit hearts improved the recovery of ventricular and coronary vascular function via NO-dependent mechanisms. Furthermore, in eNOS-KO mice the threshold for PC is higher than in controls, suggesting an important role for NO in PC (Feng *et al.*, 2000, Bell *et al.*, 2001). However, there is still strong controversy about the role of NO in classical PC against infarction (Patel *et al.*, 1993, Goto *et al.*, 1995, Nakano *et al.*, 2000).

Kinin-induced PC in humans. The important question of whether the PC effect seen in animal models also applies to humans is still unanswered (Nakano *et al.*, 2000), and there have been only two studies so far concerning BK and PC in humans. In 1997, Morris *et al.* (1997) showed that ACEi potentiated PC in human atricular trabeculae possibly through a BK-2R-mediated mechanism. Another study by Leesar *et al.* (1999) showed that a 10-minute intracoronary infusion of BK before PTCA reduced ST-segment shift in ECG during the first (the strongest effect), second and third inflation, and similarly reduced chest pain score and regional wall motion during inflation, strongly indicating a PC effect in this setting in humans.

In conclusion, ischemia and other measures that increase kinin concentrations produce the classical PC effect. The effect of kinins on the level of intracellular signaling merges with other signaling pathways producing PC, the role of NO in this signaling is still unclear.

Second window of PC – late PC

Late PC is different from classical PC regarding the triggering signals, intracellular signaling cascades and the time that its effect takes place. While the classical PC lasts 2 to 3 hours after the stimulus and protects against infarction but not against stunning, late PC lasts 3 to 4 days and protects against both infarction and stunning.

Late PC requires activation of multiple stress-response genes and a complex intracellular signaling cascade, including PKC, Src protein tyrosine kinases, NF-κB, ending in an increase in, for example inducible nitric oxide synthase (iNOS), COX-2, and aldose reductase. Several triggers can induce late PC, including heat stress,

cytokines and exercise. Importantly, the effect can be reproduced pharmacologically with clinically relevant agents, such as NO donors, adenosine receptor agonists and opioid agonists, enabling pharmacological exploitations (Bolli *et al.*, 1998 and 2000).

The first study linking BK to late PC was that by Kim *et al.* (1997), showing that a 10-minute PC in chronically instrumented dogs induced an increase in the response to acetylcholine and BK after 6 hours, reaching a maximal response at 1-2 days. An increase in the content of coronary oxygen and the production of NO metabolites was also observed. The effect could be blocked by an inhibitor of NO, suggesting a role for NO in this phenomenon. The investigators did not attempt to block the effect by using HOE-140, leaving the question of the role of kinins unanswered.

Recently, ACEi has been shown to induce late PC in instrumented pigs. ACEi alone induced a milder protective effect and, in combination with a ”milder” PC (2x2min ischemia), resulted in protection very similar to a more extensive type of PC (4x5min ischemia) alone. The investigators propose that this effect is mediated by BK (Jaberansari *et al.*, 2001).

It was also recently shown that PC in rats and rabbits either with ischemia-reperfusion or with BK infusion resulted in significant reduction of infarct size (from 50% to 30% and ~40% to ~20% respectively). The effect was fully blocked by HOE-140, confirming the role of BK and BK-2R in late PC (Ebrahim *et al.*, 2001, Kositprapa *et al.*, 2001). In conclusion, BK, in addition to classical PC, can induce a late PC effect and the effect seems to be signalled through NO.

BK-1R and PC

There are only three reports addressing the role of BK-1Rs in classical PC. In 1993, Chahine *et al.* (1993) showed that in isolated rat hearts, after 30 minutes of ischemia, the reduction of noradrenalin flow was reduced by BK and inhibited by BK-1R antagonist. This result was confirmed by Feng *et al.* (1997) and Bouchard *et al.* (1998), who also showed that BK-1R antagonists blocked the positive effect of PC on the endothelium-dependent vasodilatation.

3.4. The kininogen-kallikrein-kinin system in the pathogenesis of heart failure

In addition to the cardioprotective effects that kinins have on diseases that contribute to the development of HF (diabetes, hypertension and MI), the kinin system is also directly involved in the pathogenesis of HF. Kinins have been shown to counteract several different malfunctions in myocardial physiology, including endothelial function, myocardial oxygen consumption, cardiac function and LVH.

3.4.1. The components of the kininogen-kallikrein-kinin system in heart failure

Kinin concentrations

There are no reports estimating whether the rate of formation of kinins changes in the pathophysiology of HF. Concerning their degradation, it has been shown that BK

degradation is not changed in the SHR model of HF (Dendorfer *et al.*, 2001b). In animal models of HF the kinin levels in the blood were unchanged in several studies (Su *et al.*, 1999, Cugno *et al.*, 2000, Duncan *et al.*, 2000), but there have been no studies concerning heart tissue or kinin levels in human HF patients.

The expression of BK receptors and eNOS

There are no data concerning the expression of BK receptors at any stage of the pathogenesis of HF. However, there are studies concerning the expression of signaling cascade proteins downstream of the BK receptors. It has been shown that eNOS, the main second messenger of kinin receptors, is increased in aortic banding induced hypertrophy and also in early LVH in rats and in guinea-pigs, respectively (MacCarthy *et al.*, 2000, Barton *et al.*, 2001, Grieve *et al.*, 2001). However, during the transition from LVH into HF, the expression of both endothelial eNOS and NO bioactivity decrease (Smith *et al.*, 1996, Grieve *et al.*, 2001). In addition, COX-1 expression in the vasculature is decreased during HF (Smith *et al.*, 1996), which suggests that EDHF may be the only fully intact mediator of kinin-induced vasodilatation, as EDHF probably reaches full capacity without inhibition by NO (Brandes *et al.*, 2000). This may also explain the more significant effects shown for EDHF and less significant for NO in HF compared to the normal situation (Malmsjö *et al.*, 1999). Interestingly, BK has been shown to increase eNOS expression in normotensive rats through a BK-2R-mediated mechanism (Bachetti *et al.*, 2001).

3.4.2. Kinins and endothelial dysfunction in heart failure

The term endothelial dysfunction includes a variety of faults in the endothelium which, depending on the basic pathology, include functional impairment of vasodilating substances, such as acetylcholine and BK, or a more generalized problem of EC physiology with vasodilator receptor downregulation or impaired intracellular cascades for a vasodilatory response (Ferrari *et al.*, 1998).

The KKK seems to be one of the vasodilator systems that are impaired in endothelial dysfunction. Theoretically, the impairment could be mediated through decreased formation of kinins, a decreased amount or activity of kinin receptors on the endothelium, a leaking endothelium resulting in BK-2R stimulation directly on SMCs, causing vasoconstriction, or impairment of BK-dependent secondary mediators, such as eNOS downregulation.

BK-1Rs and BK-2Rs are present both in the coronary arteries and in the peripheral vasculature, having the potential to affect systemic blood pressure and coronary flow (Belichard *et al.*, 1996, Witherow *et al.*, 2001). In healthy dog and human hearts, it has been shown that endogenous BK participates in the *in vivo* regulation of coronary blood flow under basal and flow-stimulated conditions (Groves *et al.*, 1995, Cheng *et al.*, 1998). Indeed, lack of BK-2R, as shown in the BK-2R-KO mice, leads to impairment of flow-dependent vasodilatation. In addition, ACEis can increase kinin concentrations, directly potentiate active BK receptors on the endothelium, and possibly through these effects increase the expression of eNOS in the ECs, thus enhancing the actions of the kinin system (Pellacani *et al.*, 1994, Minshall *et al.*, 1997, Ferrari *et al.*, 1998).

It has been shown that in diabetes, hypertension and CHD, the major diseases leading or contributing to the progression of HF, BK-induced vasodilation is impaired (Kuga *et al.*, 1995, Panza *et al.*, 1995, Benacerraf *et al.*, 1999, Zhao *et al.*, 1999, Ding *et al.*, 2000, Vallejo *et al.*, 2000). Thereafter it is unlikely that the onset of HF would cause any improvement in the BK system. In addition, it has been shown that BK-induced vasodilatation is impaired in HF even in the absence of other prior diseases (Kichuk *et al.*, 1996, Straeter-Knowlen *et al.*, 1999).

Although there probably are several parallel mechanisms participating in the onset of HF, one important mechanism may be a significantly reduced amount of NO as a result of eNOS downregulation (Recchia *et al.*, 1998, Varin *et al.*, 2000). As ACEi have been shown to improve endothelial dysfunction and to increase eNOS expression by a BK-mediated mechanism, and since BK-2R upregulation induces similar effects (Auch-Schwelk *et al.*, 1995, Mancini *et al.*, 1996, Ferrari *et al.*, 1998, Straeter-Knowlen *et al.*, 1999), it is possible that downregulation of eNOS is mediated by lack of a stimulatory signal from the BK-2R. However, there are no data available on the levels of BK-R in HF. Importantly, signals causing endothelial dysfunction have been shown to impair the anti-hypertrophic effect of BK, one of the most important effects of kinins in HF (Rosenkranz *et al.*, 1999 and 2000). Some controversial findings have also been made that question the role of BK in endothelial dysfunction of the heart (Rizzoni *et al.*, 1998, Davie *et al.*, 1999, Varin *et al.*, 2000).

3.4.3 The effect of kinins on myocardial oxygen metabolism and cardiac function in heart failure

In addition to its vasodilatory action, BK has been shown to decrease the myocardial consumption of oxygen through an effect on mitochondrial respiration (Pittis *et al.*, 2000). This effect is mediated by the BK-2R and NO activation; it can be enhanced by either ACE inhibition or Ca-antagonism (Amlodipine), and has been shown to occur in both dogs and humans (Zhang X *et al.*, 1997, 1999a and 1999b). The mechanism is impaired in BK-2R-KO mice and after induction of HF in dogs, and in the latter model, ACE inhibition could at least partially correct it, suggesting that both BK and BK-2R are responsible for the effect (Xie YW *et al.*, 1996, Loke *et al.*, 2000).

Myocardial capillary density. During the development of LVH, the CMCs grow more than the capillaries, causing a decrease in capillary density. Growth of new capillaries, however, is known to be possible even in the human adult heart (Fulton *et al.*, 1965, Crisman *et al.*, 1985) and ACEis have been shown to promote capillary growth mediated by BK-2R (Unger *et al.*, 1992). BK-2R stimulation improves blood flow and increases shear stress, which has been shown to trigger capillary growth (Schölkens *et al.*, 1988, Mall *et al.*, 1990). It has been directly shown in rats, using HOE-140, and in BK-2R-KO mice that BK can promote capillary growth through BK-2R, and that the effect can be enhanced by ACE inhibition (Gohlke *et al.*, 1997, Silvestre *et al.*, 2001). Moreover, both BK-1R and BK-2R can reduce intimal thickening in coronary arteries in the mouse (Emanueli *et al.*, 2000).

With regard to cardiac function, BK has also been suggested to decrease cardiac contractility *in vitro* both in isolated myocytes and in isolated working rat hearts through NO and EDHF (Brady *et al.*, 1993, Fort *et al.*, 1993, Kitakaze *et al.*, 1995, Ou *et al.*, 1999, Rastaldo *et al.*, 2000) and increase it *in vivo* (Munch *et al.*, 1991,

Minshall *et al.*, 1994). The explanation may be that *in vivo* BK causes vasodilatation, which induces a "Gregg phenomenon", i.e. an increase in blood flow increases contractility and at the same time both NO and EDHF reduce the contractility of myocytes directly (Minshall *et al.*, 1997a). The net result would be increased contractility, with increased blood flow relative to contraction, which would offer protection for the heart from ischemia and anaerobic metabolism during HF.

Lastly, and probably through the previous mechanisms, BK has been shown to improve cardiac metabolism and improve cardiac output both at rest and during work through enhanced LV relaxation and increased contractile performance (Gohlke *et al.*, 1994a and 1994b, Yang X *et al.*, 1996, Cheng *et al.*, 1998, Hu *et al.*, 1998). Some effects were shown to act through endogenous kinins, and for others ACE inhibition was needed (Gohlke *et al.*, 1994, Trippodo *et al.*, 1995, Cheng *et al.*, 1998).

3.4.4. Kinins in left ventricular hypertrophy

LVH is the most important remodelling phenomenon taking place in the pathogenesis of HF. It also seems to be the bottleneck for the transition to clinical HF, since LVH always precedes HF. LVH is a combination of interstitial fibrosis in the myocardium and hypertrophy of the CMCs. Several signals that stimulate the production of LVH, for instance Ang II or endothelin (Oriji *et al.*, 2000, van Wamel 2001), but only recently different protective mechanisms such as the kinin system have been shown to be able to inhibit it.

The growth-regulatory effect of BK on myocardial cells

BK induces different growth-regulating effects in myocardial cells. ECs produce NO and prostaglandins, which both have been suggested to be antihypertrophic signals in the myocardium (Gallagher *et al.*, 1998, Raij *et al.*, 1998).
Cardiac fibroblasts also express BK-2R (Villarreal *et al.*, 1998) and their stimulation has been shown to inhibit EGF- and PDGF-induced DNA synthesis and induce reductions in collagen expression in the cardiac fibroblasts through the production of prostaglandins, especially PGI_2 (Patel *et al.*, 1992, McAllister *et al.*, 1993, van Zoelen *et al.*, 1994, Yu *et al.*, 1997, Gallagher *et al.*, 1998).
In vivo, CMCs are stimulated by BK in two ways, directly through their own BK-2Rs and indirectly through endothelium-derived NO. Both direct and indirect stimulation of BK-2R has been shown to inhibit the hypertrophic responses to phenylephrine and to Ang II, respectively (Ishigai *et al.*, 1997, Ritchie *et al.*, 1998a, Matoba *et al.*, 1999). Conversely, endothelial dysfunction, produced by hydrogen peroxide, decreases endothelial NO production and this inhibits the antihypertrophic effect of the kinin system on CMCs (Rosenkranz *et al.*, 2000).

The effect of endogenous bradykinin *in vivo*

There are many studies showing that kinins have an LVH-reducing effect when they are enhanced by ACEi. Linz *et al*. (1992), first showed that LVH in rats with aortic banding was reduced by ACEi treatment and that the reduction was prevented by the HOE-140. This kinin-mediated antihypertrophic effect was subsequently shown in SHR, MI and in uremic hypertensive rat models and in a dog model of LVH and HF (McDonald KM *et al.*, 1995, Harrap *et al.*, 1996, Liu Y-H *et al.*, 1997, Hu *et al.*,

1998, Amann *et al.*, 2000). Interestingly, in old SHRs, ACEis were shown to improve survival, possibly through an upregulation of eNOS and NO, and thus, improved endothelial dysfunction. Since BK-2Rs have been shown to induce eNOS expression, it seems likely that the observed upregulation of eNOS in the SHR is a BK-2R-dependent effect (Linz *et al.*, 1999, Bachetti *et al.*, 2001). In parallel to ACE inhibition, AT_1 blockade and overexpression of the human tKLK gene in rats, which both increase the concentration of endogenous kinins, have also been shown to inhibit the progression of LVH, through a BK-2R-mediated effect (Liu Y-H *et al.*, 1997, Silva *et al.*, 2000).

Since the earlier work was done using ACEi, it has been questioned whether endogenous kinins can affect the LVH without their enhancement through ACE inhibition. Indeed, the BK-2R antagonist, HOE-140, has been shown to predispose to the development of LVH after MI in the rat, suggesting a role for the endogenous kinins (Wollert *et al.*, 1997, Hu *et al.*, 1998). The antiproliferative effect of BK was stronger in reducing fibrosis than in inhibiting myocyte hypertrophy. In addition, BK-2R has been shown to inhibit the increase in myocardial intercapillary distance in uremic hypertensive rats, suggesting a reduction in LVH (Amann *et al.*, 2000).

In animal studies, the strongest evidence in support of an essential role for BK in cardiovascular physiology has come from knockout mouse models.
First, it has been shown that in the Brown-Norway-Katholiek rat, which is an inbred strain lacking the HMW kininogen, and in the BK-2R-KO mouse, the ACEi-induced cardioprotection against remodelling after MI is not operative. Therefore, it is evident that protection of the myocardium against remodelling after infarction is mediated by BK (formed by pKLK), which stimulates the BK-2R (Liu *et al.*, 2000, Yang *et al.*, 2001).

In addition, it has been shown that the BK-2R-KO mice develop HF, which cannot be explained by the mild hypertension that they have (Emanueli *et al.*, 1999). Interestingly, the tKLK-KO mice also develop cardiac dilatation and other abnormalities, but have no hypertension (Meneton *et al.*, 2001). Since the BK-1R-KO mouse has been shown to be cardiovascularly normal (Pesquero *et al.*, 2000), while BK-2R-KO mouse develops HF, it seems very likely that the BK-2R mediates the lacking cardioprotection in the tKLK-KO model.

Induction of HF by knocking out the BK-2R was later shown to be inhibitable by the AT_1 antagonist, suggesting that there is a finely tuned balance between the KKK system and the RAS (Madeddu *et al.*, 2000) which, without the protective kinin system, is destroyed and leads to unopposed action of the RAS, to LVH and to progressive HF.

However, also conflicting data exist concerning the role of the BK-2R in normal cardiovascular physiology (Rhaleb *et al.*, 1999). These results have later been suggested to depend on the influence of the different genetic backgrounds of the BK-2R strains, such as the upregulation of BK-1Rs or tKLK in the BK-2R-KO mice counteracting the lack of BK-2R (Duka *et al.*, 2001, Monti *et al.*, 2001). Similar compensations can be seen in other knockout models, such as the eNOS-KO, in which the KKK system has been shown to be upregulated as a compensatory effect, and

prostaglandins and EDHF possibly substituting for eNOS in mediating vasodilatation (Chao C *et al.*, 1996, Puybasset *et al.*, 1996, Brandes *et al.*, 2000).

Possibly the strongest evidence overall supporting the clinically relevant role of the KKK system in opposing the production of LVH came from a prospective study of human polymorphism. Healthy persons were divided into six groups according to their ACE and BK-2R gene alleles. The study showed that the amount of physiological LVH (which may differ from the pathological LVH) produced during a ten week physical training programme was indirectly proportional to the activities of BK-2R and to the activity of ACE (the more activity, the lower the kinin concentration) (Brull *et al.*, 2001). The importance of these results is underlined by earlier results from the same group that gave similar results with regard to ACE polymorphism and the production LVH, but showed that losartan, an AT_1 antagonist, had no effect on the production of LVH (Myerson *et al.*, 2001).

AIMS OF THE PRESENT STUDY

The aim of the study was to investigate the regulation of kinin metabolism in the circulation and in the normal and failing heart tissue. More specifically the aims were:

1. To clarify which enzymes are responsible for the kinin degradation in the human plasma.

2. To find out which enzymes are responsible for the kinin degradation in the normal and failing human heart tissue.

3. To investigate the expression of BK receptors in an animal model of LVH and HF.

4. To investigate the expression of BK receptors in the normal and failing human heart tissue.

MATERIALS AND METHODS

1. Kinin degradation

1.1. Acquiring human heart samples (I, III)

Normal heart samples (n=6) were obtained from left ventricles of organ donors with no history of cardiac disease, who had been excluded from organ donation because of age, body size, or blood type incompatibilities. Failing left ventricles were harvested at the time of cardiac transplantation from 13 patients with end-stage HF (New York Heart Association functional class III to IV) due to either IDC (n=7) or CHD (n=6) at the University Central Hospital, Helsinki, Finland, or at the General Hospital, Vienna, Austria. All the patients were treated with a combination of drugs including β-blocker, ACEis, loop diuretics, digoxin and spironlactone. After excision, the heart tissue was immediately frozen in liquid nitrogen and stored at -70°C. Myocardium devoid of visible scar tissue was used in the experiments. (For I: 6, 4 and 5 first samples of the above patient groups were used and for III: all 19 samples were used) The clinical characteristics of the patients in this study are shown in Table 1 (I). The Internal Review Committees at the corresponding hospitals approved the use of human heart samples.

1.2. Preparation of human cardiac membranes for enzymologic measurements (I)

The heart tissue piece was homogenized in PBS at 4°C (100 mg tissue/ml) with an Ultra-Turrax T25 homogenizer (IKA-Labortechnik, Staufen, Germany) at 13,500 rpm for 1 minute. Cardiac membranes were prepared by centrifugation of the homogenates at 40,000 x g for 30 minutes at 4°C, as described by Urata *et al.* (1990). The sediments were resuspended and recentrifuged, and finally resuspended in PBS and stored at -70°C. The concentration of each cardiac membrane preparation is expressed in terms of its protein concentration. Protein was determined after solubilization with Triton-X-100 (Hatzoglou *et al.*, 1992) by the method of Lowry, with bovine serum albumin as standard (Lowry *et al.*, 1951). The protein concentrations of the different preparations varied between 0.7 and 1.5 mg/ml.

1.3. Preparation of human plasma (II)

Human blood (5 ml) was withdrawn by venipuncture in tubes containing 100 IU of dalteparin (final concentration 20 IU/ml). The anticoagulated blood was centrifuged at 1500 x g for 10 minutes at room temperature, after which the plasma was separated and stored at -20C. Plasma was prepared from 10 apparently healthy persons, 5 males and 5 females, 24 to 52 years of age. All plasmas were used within 2 days of preparation.

1.4. Determination of kinin degradation (I, II)

(I) The standard assay was conducted at 37°C in 50 µl of PBS (137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 0.9 mmol/l $CaCl_2$, 1.1 mmol/l KH_2PO_4, 0.5 mmol/l $MgCl_2$, pH 7.3, from Gibco), containing cardiac membranes (5 µg of protein), 5 nmol of synthetic kinins (Bachem), and the indicated concentrations of inhibitors

(NEP inhibitor, NEPi, SCH 39370 was a kind gift from Schering-Plough, the rest were acquired from Sigma). After incubation for the indicated times, the reactions were stopped by adding 300 µl of ice-cold ethanol, and the preparations were incubated at 4°C for a further 30 minutes to precipitate proteins. Finally, the mixtures were centrifuged at 15,000 x g for 10 minutes at 4°C to sediment the proteins. The supernatants were then collected for peptide analysis by reverse-phase high-performance liquid chromatography (RP-HPLC).

(II) The other standard assay was conducted at 37°C in 25 µl of PBS (137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 0.9 mmol/l $CaCl_2$, 1.1 mmol/l KH_2PO_4, 0.5 mmol/l $MgCl_2$, pH 7.3), containing 1 mg/ml of bovine serum albumin (BSA), 20 IU/ml of dalteparin, 2.5 µl of plasma, the indicated concentrations of enzyme inhibitors, and 2.5 pmol of 3H-BK (final concentration 100 nmol/l). For the experiments, the labeled BK was diluted with unlabeled BK to give the specific activities indicated in the figure and table legends. For the experiment measuring the effect of decreasing substrate concentration on the BK degradation pattern, the following BK concentrations/incubation times were used: for BK concentrations 30 to 300 nmol/l the incubation time was 10 minutes, for 1 to 10 µmol/l 20 minutes, and for 30 to 100 µmol/l 80 minutes.

1.5. Determination of ACE activity (I)

ACE activity in the cardiac membranes was measured with FAPGG (Kokkonen *et al.*, 1997) as substrate. The degradation of FAPGG to FAP by the ACE was monitored by RP-HPLC. The standard assay was conducted at 37°C in 50 µl of PBS containing cardiac membranes (10 µg of protein) and 5 nmol of FAPGG. After incubation for 2 hours, the reactions were stopped with ice-cold ethanol, and the samples were prepared for RP-HPLC analysis as described below for kinin peptides.

1.6. Reverse-phase high-performance liquid chromatography analysis (I, II)

For RP-HPLC analysis, the supernatants containing kinin peptides or FAPGG were evaporated to dryness and then dissolved in 100 µl of 0.1% trifluoroacetic acid. The samples were analyzed by RP-HPLC as described (Kokkonen *et al.*, 1997). Kinin peptides were identified by comparing the retention times of the peaks with those of synthetic standards, and by N-terminal sequence analysis of the eluted material. Formation of kinin peptides or FAP was quantified by measuring peak area or peak height relative to known standards. The results are expressed as nmol of kinin peptides or FAP formed per minute per mg of cardiac membrane protein.

With 3H-BK (II) fractions of 250 µl (30 sec) from the RP-HPLC eluate were collected and measured for their 3H-radioactivity. Kinin peptides were identified by comparing the retention times of the peaks with those of synthetic standards, and by N-terminal sequence analysis of the eluted material. Formation of kinin peptides was quantitated by counting the radioactivity in each peak area. The results are expressed as nmol of BK peptides formed per minute per liter of plasma. The recoveries of the eluted 3H-labeled material averaged over 90% of the radioactivity applied to the column.

1.7. N-terminal sequence analysis of kinin peptides (I, II)

The kinin peptide fractions obtained from the RP-HPLC analysis were subjected to an automatic sequence analysis with an Applied Biosystems Procise 494 protein sequencing system and a Model 610 data analysis system.

2. Kinin receptors (III, IV)

2.1. Experimental animal preparation (IV)

We used spontaneously hypertensive male rats (SHR) of the Okamoto-Aoki strain, male Wistar-Kyoto rats (WKY), and 10- to 12-week-old male Sprague-Dawley (SD) rats from the Center for Experimental Animals at the University of Oulu, Finland. The SHR strain was originally obtained from Mollegaards Avslaboratorium, Skensved, Denmark. SHRs were studied at the ages of 2 weeks (prehypertensive stage), 3 months (early stage of established hypertension and hypertrophy), 12 months (chronic stage of hypertension and hypertrophy), and 20 months (transition stage to HF). The experimental procedures for the animals were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the University of Oulu, Finland and the Animal Experimentation Committee of the Wihuri Research Institute, Helsinki, Finland. After decapitation, LV free walls were removed. The tissues were snap frozen in liquid nitrogen and stored at -70°C for molecular, biochemical, and histological analysis.

2.2. Aortic banding in SD rats (IV)

SD rats were anesthetized intraperitoneally (i.p.) with 0.26 mg/kg fentanyl citrate, 8.25 mg/kg fluanisone, and 4.1 mg/kg midazolame. The aorta was exposed through a midline abdominal incision, freed by blunt dissection at the level of the renal arteries and isolated with a silk suture positioned between the right and left renal arteries. A 24-G needle was placed on the top of the aorta and a previously made suture was tightened against the needle, which was then promptly removed to produce a predefined constriction across the abdominal aorta. Sham-operated animals underwent the same surgical procedure, except that the ligature was not tightened against the needle. After the operation, the rats were housed individually in experimental cages and had free access to food and water. Buprenorphin (0.3 mg/kg s.c.) was used during the first day for postsurgical analgesia. Hemodynamic measurements were made in the anesthetized animals. The rats were cannulated with PE-50 catheters inserted into the right femoral and the right carotid arteries for measurement of blood pressures below and above the aortic banding. Both catheters were exteriorized behind the neck and attached to pressure transducers (Model MP-15, Micron Instruments, Los Angeles, California, USA) and a Grass polygraph (Model 7DA, Grass Instruments, Quincey, Massachusetts (MA), USA) and blood pressure was registered for 60 minutes.

2.3. Angiotensin II infusion in SD rats (IV)

Ang II was administered to conscious rats by SC osmotic minipumps (Alzet 1003D and 2002, B&K Universal AB, Sollentuna, Sweden) for 2 weeks. For telemetric monitoring of mean arterial pressure, SD rats were anesthetized as described above

and fitted with a catheter coupled to a sensor and transmitter (TA11PA-C40, DataSciences, Minneapolis, MN) in the abdominal aorta below the renal arteries. On the 7th day after implantation, the rats received either Ang II (33.3 µg/kg/h) or 0.9 % NaCl for 2 weeks. Blood pressure was continuously monitored throughout the infusion and recovery periods.

2.4. Echocardiography in rats (IV)

Transthoracic echocardiograms were performed, using a commercially available Acuson Ultrasound System (Sequoia^{TM} 512) and a 15-MHz linear transducer (15L8) (Acuson, MountainView, California, USA). Before examination, the rats were sedated with ketamine 50 mg/kg and xylazine 10 mg/kg and their chests were shaved. The rats were placed in the supine position, normal body temperature being maintained during the examination by a warming pad and lamp. Using two-dimensional imaging, a short axis view of the left ventricle at the level of the papillary muscles was obtained and two-dimensionally guided M-mode recordings through the anterior and posterior walls of the left ventricle were obtained. LV end-systolic (LVESD) and end-diastolic (LVEDD) dimensions, as well as interventricular septum (IVS) and posterior wall (PW) thicknesses, were measured from the M-mode tracings. The LV shortening fraction (LVFS) was calculated from the M-mode LV dimensions, using the following equation: LVFS (%) = {(LVEDD-LVESD) / LVEDD} x 100. The ejection fraction (EF) was also calculated from the M-mode LV dimensions, using the equation: EF (%) = $\{(LVEDD)^3 – (LVESD)^3 / LVEDD^3\}$ x 100. For evaluation of LV diastolic function, mitral flow was recorded from an apical four-chamber view. The peak flow velocities of the early rapid diastolic filling wave (E) and the late diastolic filling wave (A) were measured.

2.5. Detection of BK-2R and BK-1R mRNA by competitive RT-PCR (III, IV)

Total RNA was isolated from human or rat heart samples, using an ultra-pure TRIzol reagent (GIBCO BRL) and a RNeasy Mini Kit (QIAGEN). One microgram of purified total RNA was transcribed into cDNA, using a Superscript TM pre-amplification system (GIBCO BRL). Competitive RT-PCR was performed in 25 µl of standard PCR buffer containing 1 µl of the RT reaction mixture, 25 pmol of sense and antisense primers, 100 µmol/l of each deoxynucleotide, 0.5 units of Taq DNA polymerase (Roche), and competitor DNA.
The primers were as follows:

III:
BK-2R: 5'-CACCATCTCCAACAACTTCG (S), 5'-GGTAGCTGATGACACAAGC G (AS); **GAPDH**: 5'-ACCACAGTCCATGCCATCAC (S), 5'-TCCACCACCCTGTT GCTGTA (AS).
The competitor DNA for the BK-2R was obtained by inserting an 129 bp external DNA fragment into the SacI site.

IV.
BK-1R: 5'-TTAACTGGCCTTTCGGAGCC (S), 5'-CCACAATCCTTGCAAAGTGC (AS), **BK-2R**: 5'-CACCATCTCCAACAACTTCG (S), 5'-GGTAGCTGATGACACA AGCG (AS); **GAPDH**: 5'-ACCACAGTCCATGCCATCAC (S), 5'-TCCACCACCC TGTTGCTGTA (AS).

The competitor DNA for BK-1Rs and BK-2Rs was obtained by inserting an 129 bp external DNA fragment into a SmaI and SacI site, respectively.

The PCR product was verified, by DNA sequencing, to represent the corresponding target. The use of equal amounts of mRNA in the RT-PCR assays was confirmed by analyzing the expression levels of a housekeeping gene, GAPDH (data not shown). The PCR products were quantified with a Gel Doc 2000 gel documentation system (Bio-Rad) and the logarithm of the target-to-competitor ratio was plotted against the logarithm of the competitor DNA molecules (Shiota *et al.*, 1998).

2.6. Detection of BK-2Rs and BK-1Rs by Western blotting (III, IV)

Triton-X-100 extracts were prepared from normal and failing heart tissue, subjected to SDS-PAGE and electroblotted onto nitrocellulose filters (Lindstedt *et al.*, 1997).

III. The BK-1Rs were detected by Western blotting, using a polyclonal BK-1R antibody (0.25 mg/l, prepared as previously described by Raidoo *et al.* (1997), a peroxidase-conjugated goat anti-rabbit antibody (0.001 mg/l, DAKO) and enhanced chemiluminescence (Amersham Pharmacia Biotech) as recommended by the manufacturer.
The BK-2Rs were detected by Western blotting using a monoclonal BK-2R antibody (0.25 mg/l, Transduction Laboratories), a peroxidase conjugated goat anti-mouse antibody (0.001 mg/l, DAKO) and enhanced chemiluminescence (Amersham Pharmacia Biotech) as recommended by the manufacturer.

IV: The BK-2Rs were detected by Western blotting, using a monoclonal BK-2R antibody (0.25 mg/l, Transduction Laboratories), a peroxidase-conjugated goat anti-mouse antibody (0.001 mg/l, DAKO) and enhanced chemiluminescence (Amersham Pharmacia Biotech) as recommended by the manufacturer.

The blots were quantified with a Gel Doc 2000 gel documentation system (Bio-Rad) and the level of BK-1R/2R expression in failing hearts was expressed as a percentage of the control.

2.7. Histo- and immunohistochemical staining of normal and failing hearts (III, IV)

Frozen sections of WKY, SHR and human hearts of different ages were stained for fibrotic tissue, using a commercial Masson trichrome staining kit (Accustain^{TM}, Sigma Diagnostics), and for BK-2Rs, using a monoclonal anti-BK-2R antibody (5 mg/l, Transduction Laboratories). Equal amounts of nonimmune serum or PBS were used to control for the specificity of the primary and secondary antibodies. Control sections were further stained for ECs with a peroxidase-conjugated rabbit polyclonal anti-von Willebrand factor antibody (26 mg/l, DAKO). The density of the Masson trichrome staining (blue color=fibrosis; red color=myocytes) and of the antibody-peroxidase generated color (reddish-brown) in the stained sections was quantitated, using the Image-Pro Plus (version 4.0/4.1) image analysis system (Media Cybernetics).

3. Statistical analyses (I-IV)

I: The results are expressed as means of ±SEM. Differences between groups were tested by using the Kruskal-Wallis test and a P value of <0.05 was considered statistically significant.

II: The results are expressed as means of ±SEM, and a P value of <0.05 was considered statistically significant.
In Table I (II), differences between two groups (male and female) were analyzed using a logistic regression model. In this model, the proportion of BK-(1-5) + BK-(1-7) (=ACE activity) over BK-(1-5) + BK-(1-7) + BK-(1-8) (=ACE+CPN activity) was used as the dependent variable, whereas sex and age were used as explanatory variables.

III: Data are expressed as mean values (±SEM). All three patient groups showed normal distributions when analyzed with the Shapiro-Wilks W-test and comparisons between the groups of normal and failing hearts were made using a two-way ANOVA with Dunnett´s post hoc test. Simple linear regression analysis was used to determine the relation between 2 variables. Statistical significance was accepted at $P<0.05$.

IV: To compare the mean values (±SEM) between the groups, the data was analyzed using the unpaired two-tailed Student t test. Statistical significance was accepted at $P<0.05$.

RESULTS

1. Degradation of kinins (I, II)

1.1. Human heart tissue (I)

1.1.1. Degradation of kallidin by human cardiac membranes (I)

We first studied the degradation of the two kinins, KD and BK, by human cardiac membranes as a function of time. For this purpose, KD was incubated with cardiac membranes derived from a normal heart for 2 hours at 37˚C. In RP-HPLC analysis of KD-derived peptides one major and three others eluted. These were identified as a) BK and its degradation product b) BK-(1-7), and two degradation products of KD c) KD-(1-8) and d) KD-(6-10).

The rates of formation of the major KD-derived metabolite BK and also KD-(6-10) and KD-(1-8) were linear for 90 minutes. After an initial lag phase of 30 minutes, the formation of the secondary degradation product BK-(1-7) proceeded linearly.

1.1.2. Degradation of bradykinin by human cardiac membranes (I)

BK was incubated similarly to KD with cardiac membranes. Only one major degradation product BK-(1-7), eluted, and its formation was linear for the whole 90-minute incubation period.

1.1.3. Comparison of normal and failing hearts in their ability to degrade KD and BK (I)

The normal and the failing hearts did not differ significantly in their abilities to degrade KD and BK. Nor was a significant difference observed in the abilities of CHD and IDC hearts to degrade KD and BK (Table 1, I).

1.1.4. Inhibition of kallidin and bradykinin degradation by enzyme inhibitors (I)

The similar degradation patterns of kinins by cardiac membranes from normal and failing hearts suggested that the enzymes responsible for the degradation were the same in every membrane preparation. To study the contribution of the enzymes potentially involved, the degradation of KD and of BK were studied in the presence of various enzyme inhibitors. With all three different patient populations, the results were closely similar. Conversion of KD to BK was effectively inhibited by amastatin, a widely used inhibitor of aminopeptidases A and M (Orawski *et al.*, 1989). In addition, conversion of KD to BK was inhibited to about 80% by bestatin (300 µmol/l), an inhibitor of APM (EC 3.4.11.2) (Orawski *et al.*, 1989, Palmieri *et al.*, 1989). Since bestatin is not absolutely specific for APM, this bestatin-inhibitable activity in human cardiac membranes will hereafter be referred to as APM-like activity. Formation of KD-(6-10), KD-(1-8), and BK-(1-7) was effectively inhibited by phosphoramidon, a widely used but not absolutely specific inhibitor of neutral endopeptidase (NEP; EC 3.4.24.11) (Orawski *et al.*, 1989), and by SCH 39370, a specific NEPi (Sybertz *et al.*, 1989).

Further, formation of BK-(1-7) was fully inhibited by amastatin and bestatin, indicating that this peptide was derived from BK. In sharp contrast, captopril had no significant effect on the formation of any of the peptides studied.
The above experiments were repeated with BK as substrate. Unexpectedly, captopril, a specific ACEi, had no effect on BK-(1-7) formation. The experiment was repeated with another ACEi, lisinopril (10 µmol/l), and again no inhibition was found. However, formation of BK-(1-7) from BK was effectively (80 - 90%) inhibited by phosphoramidon, a widely used inhibitor of NEP (Orawski *et al.*, 1989). Phosphoramidon is not entirely specific for NEP, because, at high concentrations, it may also inhibit endothelin-converting enzyme (Fujita *et al.*, 1994). Therefore we tested the effect of the specific NEPi, SCH 39370 (Sybertz *et al.*, 1989), on the formation of BK-(1-7). The results were very similar to those obtained with phosphoramidon. In addition, we were able to show that, at the concentrations used, phosphoramidon and SCH 39370 were unable to inhibit BK degradation by purified rabbit ACE, and that captopril was unable to inhibit degradation of BK by purified rabbit NEP (data not shown). In contrast, neither amastatin (10µmol/l) nor bestatin (300µmol/l; data not shown), two aminopeptidase inhibitors, had any effect on BK-(1-7) formation. These findings showed that in the cardiac membranes, the conversion of BK to BK-(1-7) was due mostly to NEP, which is known to hydrolyze the Pro^{7}-Phe^{8} bond in BK, producing the same metabolite, BK-(1-7), as ACE (Skidgel *et al.*, 1984).

1.1.5. The role of ACE in heart tissue bradykinin metabolism (I)

The above results suggested that ACE (EC 3.4.15.1) plays no significant role in the metabolism of kinins in the membrane fraction of heart tissue. The patients were all under chronic ACEi therapy, which, in the *in vitro* assays, may have inhibited the ACE activity in the cardiac membranes derived from their hearts. To rule out this possibility, we compared the ACE activities in the cardiac membranes of normal (n=5) and failing hearts from patients with chronic ACEi therapy (n = 10), and in cardiac membranes from patients not treated with ACEis (n = 4). ACE activity in cardiac membranes can be measured specifically with furanacryloyl-Phe-Gly-Gly (FAPGG) as substrate (Kokkonen *et al.*, 1997). As shown in table 4 (I) the cardiac ACE activities of patients receiving ACEis were not lower than those of patients not receiving ACEis or those of normal subjects. Rather, the ACE activities of patients receiving ACEis were somewhat higher, but the difference did not reach statistical significance. The degradation of FAPGG was strongly inhibited by captopril (10 µmol/l), but not by phosphoramidon (1 µmol/l), confirming the presence of ACE activity in the preparations. This finding accords with that of Urata *et al.* (1990), who showed similar ACE activities in normal and failing hearts derived from patients with or without chronic ACEi therapy.

1.2. Human plasma (II)

1.2.1. Degradation of bradykinin by human plasma (II)

We first studied degradation of BK by human plasma as a function of time. For this purpose, ^{3}H-BK was incubated with plasma derived from a healthy person at 37°C. Figure 2 (II) shows a typical RP-HPLC analysis of ^{3}H-BK-derived peptides After incubation for 8 minutes in addition to BK, three other peptide peaks eluted. N-

terminal sequence analysis disclosed that the major peptide was BK, and that the other peptides were three degradation products of BK: BK-(1-5), BK-(1-7), and BK-(1-8). ^{3}H-BK was rapidly degraded by human plasma and this degradation was closely followed by linear formation of the three degradation products, BK-(1-5), BK-(1-7) and BK-(1-8). It seems evident that degradation of BK leads to accumulation of a pentapeptide, BK-(1-5).
We then tested the degradation of BK by plasma prepared from healthy persons (n = 10; 5 males and 5 females). In all the plasmas tested (n = 10; 5 males and 5 females), the major degradation products were BK-(1-5) and BK-(1-7), and BK-(1-8) was a minor degradation product (total average 0.88 nmol/min/l). The plasmas derived from males and females did not differ significantly in their ability to degrade BK. In additional experiments, we found that the degradation of BK was similar in citrated plasma and in serum, ruling out the possibility of an effect of anti-coagulation on the degrading enzymes.

1.2.2. Inhibition of bradykinin degradation by enzyme inhibitors (II)

The similar degradation patterns of BK in every plasma sample suggested that the same enzymes were responsible for the degradation in all these samples. To study the contribution of the enzymes potentially involved, the degradation of BK was studied in the presence of various enzyme inhibitors.
Conversion of BK to BK-(1-5) and BK-(1-7) was effectively inhibited by captopril, a specific ACEi. In contrast, captopril did not inhibit the formation of BK-(1-8). However, formation of BK-(1-8) was effectively inhibited by DL-2-mercaptomethyl-3-guanidino ethylthiopropanoic acid (MGEA), a widely used, but not fully specific, inhibitor of CPN (EC 3.4.17.3) (Sheikh and Kaplan, 1989). MGEA did not inhibit the formation of BK-(1-5) or BK-(1-7). Since MGEA is not fully specific for CPN, this MGEA-inhibitable activity in human plasma will hereafter be referred to as CPN-like activity. In additional experiments we found that SCH 39370, the specific inhibitor of the neutral endopeptidase (NEP) (Stewart *et al.*, 1981) had no effect on the formation of BK-(1-5), BK-(1-7), or BK-(1-8), indicating that NEP is not involved in BK metabolism in plasma. These findings demonstrated that, in human plasma, the conversion of BK to BK-(1-7) and BK-(1-7) to BK-(1-5) was mediated by ACE. Conversion of BK to BK-(1-8) was mediated by CPN-like activity.

1.2.3. The role of CPN in bradykinin metabolism (II)

The above results suggested that CPN-like activity plays an insignificant role in the metabolism of kinins in human plasma, although in several studies it has been reported to be the major BK-degrading enzyme in plasma and serum (Levesque *et al.*, 1993, Schremmer-Danninger *et al.*, 1996). In these studies, however, the BK concentration has been well above the K_m values of the competing enzymes ACE and CPN. Since the physiological concentrations of kinins in plasma are well below the K_m values of the competing enzymes and BK has a higher affinity for ACE than for CPN (Skidgel *et al.*, 1988, Hasan *et al.*, 1996), we suspected that the BK concentration used affected the degradation profile of BK by plasma. To test this hypothesis, we incubated plasma with varying concentrations of ^{3}H-BK ranging from 30 nmol/l to 100 µM. As shown in Figure 4 (II), at the high BK concentration of 100 µmol/l, the major (over 90%) BK-degrading enzyme was CPN-like activity. With decreasing BK concentrations, there was a gradual shift in the relative activities of the

enzymes in favor of ACE over CPN-like activity. At a BK concentration of 100 nmol/l, which was used throughout this study, the major (over 90%) BK-degrading enzyme was ACE.

2. The myocardial expression of BK receptors in heart failure (III, IV)

2.1. BK receptors in human hearts (III)

2.1.1. Expression of BK-2R mRNA in normal and failing human hearts (III)

Left ventricles of normal and failing human hearts were analyzed for their mRNA levels of BK-2Rs by competitive RT-PCR. The mRNA levels of the cardioprotective BK-2Rs were found to be significantly decreased in the hearts suffering from either IDC (30% of control, P=0.003, middle row) or CHD (38% of control, P=0.01), as compared with normal hearts. The observed down-regulation of BK-2Rs in the failing hearts was not due to a local reduction in cell number, i.e., to a reduced total amount of mRNA, since the competitive RT-PCR assay was standardized to the expression level of a housekeeping gene, GAPDH. Furthermore, the observed down-regulation was specific for the BK-2Rs, since the expression of other members of the G protein-coupled receptor superfamily were either induced (AT-1Rs) or unaffected (AT-2Rs) in the failing hearts. In addition, incubating human coronary artery ECs, which express BK-2Rs in a constitutive manner, with excess BK (10 nmol/l) and an ACEi for 18 hours did not affect their levels of BK-2R expression.

2.1.2. BK-2R expression and age in human hearts (III)

Interestingly, on analyzing the data by simple linear regression analysis in the group of normal hearts, a strong positive correlation ($r=0.827$; $P<0.05$) was found to be present between the level of BK-2R mRNA expression and the age of the patients. This result suggests that normal hearts adapt to age-related changes by increasing their expression of BK-2Rs. An excellent positive correlation ($r=0.951$; $P<0.001$) was also seen within the group of IDC hearts between the level of BK-2R mRNA expression and the age of the patients. However, the relative increase in BK-2R expression was significantly lower in the IDC hearts than in the normal hearts. On account of the nature of the disease, all the CHD hearts were from patients older (>50 years) than those in the normal group, making it improbable to find a correlation. In fact, no correlation was found between BK-2Rs and age within this group.

2.1.3. BK-2R protein in normal and failing human hearts (III)

Like the mRNA levels, the protein levels of BK-2Rs were significantly reduced both in the IDC (45% of control, P=0.002) and in the CHD (62% of control, P=0.04) hearts, as compared with the normal hearts. To further analyze the distribution of BK-2Rs in normal and failing hearts, frozen tissue sections obtained from the left ventricles of normal, IDC, and CHD hearts were immunostained for BK-2Rs. The receptors were evenly distributed throughout the myocardium. In keeping with the above results, positive staining for BK-2Rs was clearly less in both IDC (42 % of control) and CHD (65 % of control) hearts as compared with normal hearts (100 %). By staining serial tissue sections with Masson´s trichrome and quantifying the level of

fibrosis, we found that the observed reduction in BK-2R expression in the failing hearts was not associated with the degree of fibrosis. Indeed, although the IDC samples had a significantly higher degree of fibrosis (20.6±1.7 %; $P<0.05$) than the normal samples (16.0±2.9 %), the degree of fibrosis in the CHD samples (20.3±8.3 %) did not significantly differ from that in the normal myocardium (16.0±2.9 %).

2.1.4. Localization of BK-2Rs and expression pattern in diseased and normal human hearts (III)

To localize the BK-2R within the myocardial cell population, we immunostained frozen tissue sections of left ventricles, using specific antibodies for BK-2Rs and specific markers of the individual myocardial cell types. All the major myocardial cell types, i.e. ECs, myocytes, fibroblasts and SMCs, were found to express BK-2Rs. The expression of BK-2Rs in ECs, SMCs and fibroblasts was further verified by cell-specific staining of serial sections of normal hearts. Interestingly, on grossly comparing the BK-2R positive cells in normal and failing hearts, we found no apparent differences in their cell-specific BK-2R expression pattern.

2.1.5. E-NOS expression in normal and failing human hearts (III)

Since the physiological effects of BK, including vasodilatation and inhibition of myocardial growth, are largely mediated through BK-2R stimulated expression and activation of eNOS (Hornig *et al.*, 1997, Bachetti *et al.*, 2001), we measured the expression level of eNOS in normal and failing hearts. The observed decrease in BK-2R expression in IDC and CHD hearts was clearly associated with a decrease in eNOS expression in IDC (47% of control) and CHD (30% of control) hearts.

2.2. BK-receptors in rat hearts (IV)

2.2.1. Expression of BK receptors in rat models of pressure overload (IV)

To study the mechanisms by which BK receptors may be regulated during the progression of LV hypertrophy, we examined two rat models of acute/subacute pressure overload, aortic banding and administration of Ang II. Banding of the abdominal aorta in SD rats led to a significant increase in their mean arterial pressure (MAP, after 12 hrs: 120±9 mmHg vs. 94±4 in sham-operated rats, and after 3 days: 111±4 mmHg vs. 90±5 mmHg in sham-operated rats; n=6) and to the onset of LV hypertrophy (LVW/BW, after 12 hrs: 2.24±0.09 mg/g vs. 2.13±0.06 mg/g in sham-operated rats and after 3 days: 2.50±0.07 mg/g vs. 2.36±0.15 mg/g in sham-operated rats). Interestingly, when left ventricles of banded and sham-operated SD rats were compared, we observed a rapid initial increase in BK-2R mRNA expression, which was already evident at 12 hrs (1.8-fold, $P<0.05$) and remained higher (3.1-fold, $P<0.05$) for 3 days after the banding procedure (Fig. 1B, IV). In contrast, the expression of BK-1Rs was not affected by the aortic banding procedure (Fig. 1A, IV).

Infusion of Ang II for 2 weeks into normotensive SD rats resulted in a cardiac pressure overload (MAP: 185±9 mmHg vs. 90±11 mmHg in the sham-operated animals; n=6), LV hypertrophy (LVW/BW: 2.78±0.145 vs. 2.43±0.004 mg/g in the sham-operated animals, $P<0.05$), but did not cause a statistically significant change in ventricular BK-2R mRNA levels. However there was a tendency of increased BK-2R

levels (Fig. 1D, IV). In contrast to BK-2R expression, no differences in BK-1R expression were observed between the infused and control animals (Fig. 1C, IV). The results suggest that BK-2R expression is increased in LVs as a consequence of acute pressure overload.

2.2.2. Left ventricular hypertrophy in SHRs and WKY rats (IV)

The SHR strain is a useful experimental model of essential hypertension and HF, (Boluyt *et al.*, 1995) in which the mean arterial pressure (MAP) is already significantly elevated at the age of 3 months (154±3 mmHg in SHR, n=8 vs. 132±5 mmHg in WKY, n=8), and continues to increase with age (164±9 mmHg in 20-month-old SHRs, n=8 vs. 108±7 mmHg in age-matched WKYs, n=8). As shown in Table I (IV), the LVwt/BW ratio, as an index of LV hypertrophy, gradually increases with age, being already significantly higher in the SHRs at the age of 3 months. Furthermore, the 20-month-old SHRs exhibited clinical evidence of HF, including pleural and pericardial effusions.

2.2.3. Echocardiography of SHRs and WKY rats (IV)

Structural and functional properties of 12- and 20-month-old SHR hearts and their age-matched WKY hearts were compared. In accordance with the findings presented in Table I (IV), the septal (IVS) and posterior wall (PW) thicknesses were significantly increased (IVS: 2.6±0.1 vs. 2.2±0.1, $P<0.001$; PW: 2.6±0.1 vs. 2.1±0.1, $P<0.001$) in the 20-month-old SHRs, indicating LV hypertrophy (Table II, IV). However, the LV size (LVEDD) and systolic function (LVFS, LVEF) in 20-month-old SHRs did not differ from those of 12-month-old SHRs. In contrast, there was a significant increase in the mitral E to A wave ratio (E/A: 4.8±0.6 vs. 2.4±0.2, $P<0.001$) in the 20-month-old SHRs, indicating severe diastolic dysfunction. None of the above echochardiographic changes occurred in the age-matched WKY rats.

2.2.4. Expression of BK-receptor mRNA in SHRs and WKY rats (IV)

The mRNA levels of the BK receptors in the left ventricles of SHRs and WKYs were analyzed by competitive RT-PCR. As shown in Figure 2A (IV), the expression of BK-1Rs was highest in the 3-month-old WKYs and SHRs, and then slowly decreased with age. The mRNA levels of BK-1Rs were significantly lower in 3- and 12-month-old SHRs than in age-matched WKY rats, but significantly higher in the failing SHR hearts at 20 months). Interestingly, the mRNA levels of the cardioprotective BK-2Rs (Fig. 2B, IV) were found to be significantly ($P<0.05$) increased in the 12- and 20-month-old SHR hearts (2.9-fold and 3-fold, respectively), as compared with age-matched WKY hearts.

2.2.5. BK-receptor protein expression in SHRs and WKY rats (IV)

To analyze the expression of BK-receptor protein, the SHR and WKY hearts were subjected to Western blot analysis. The protein levels of BK-1Rs were found to be very low at the ages between 2 weeks and 12 months, and there was no significant difference between SHRs and WKYs. However, in the failing hearts of the SHRs (20 months), the BK-1R levels were higher than those of the age-matched WKYs (Fig. 3A, IV). In contrast to the BK-1R, the BK-2R protein was significantly increased in

the 12-month-old SHRs (1.8-fold), and significantly decreased in the 20-month-old SHRs, being only 37% ($P<0.05$) of the age-matched WKYs (Fig. 2B, IV). Thus, there was a remarkable down-regulation of BK-2R protein expression in the LVs of the failing SHRs during the transition from compensated hypertrophy to HF.

2.2.6. Cellular distribution of BK-2Rs in SHRs (IV)

To analyze the cellular distribution of BK-2Rs, frozen tissue sections obtained from left ventricles of SHR hearts were immunostained for BK-2Rs. In Figure 4 (IV), myocardia from SHRs of different ages are shown, and each panel contains a middle-sized intramyocardial coronary vessel. By staining the sections with an antibody against von Willebrand factor (right panels), the endothelial layer of the vessels is identified. As shown in Panel E, in 12-month-old SHRs, the BK-2Rs (white arrows) were expressed in ECs (compare with Panel F, black arrows), and also in adventitial cells, most likely fibroblasts (Panel E, white arrowheads). Apparently, little BK-2Rs were present in the layer between the endothelium and the adventitia, i.e. in the medial SMCs. Similarly, in the tissue surrounding the vessel, i.e. in CMCs, practically no positive staining for the BK-2Rs was present. The observed increase in BK-2R expression in the aging SHRs at 12 months, as shown in Figure 3B (IV), followed by a decrease in the receptor level in failing SHRs at 20 months, appeared to be specific for the ECs (Fig. 4, IV, left panels, white arrows; right panels, black arrows).

2.2.7. Development of fibrosis in SHR and WKY rats (IV)

When serial myocardial sections were stained with Masson´s trichrome (Fig. 5, IV, panels A to H), we found that the degree of fibrosis (blue staining) at the prehypertensive stage (2 weeks) and at an early stage of hypertrophy (3 months) was very low and did not differ significantly, irrespective of age and strain. Interestingly, also, at the stage of compensated hypertrophy (12 months), the degree of fibrosis did not differ significantly between SHRs and WKYs. In striking contrast, during the transition from compensated hypertrophy (12 months) to HF (20 months) the degree of fibrosis (panel H, blue staining) in the myocardium of the SHRs increased dramatically. When quantified by an image analysis system, the increase was found to be about 10-fold (panel I, right bars, figure 5 (IV)). In summary, we could show that a loss of BK-2R expression (63%, see Fig. 3B, IV) and an increase in BK-1R expression (~1.7-fold, see Fig. 3A, IV) were associated with a significant increase in myocardial fibrosis.

DISCUSSION

1. The local kininogen-kallikrein-kinin system in the heart and the circulation (I, II).

In recent years a local KKK system has been found in many organs, such as the heart and adrenals (Nolly *et al.*, 1992, Nolly *et al.*, 1993). The two determinants of the extent of the actions of the KKK system are 1. the kinin concentration, controlled by formation and degradation and 2. the number of active kinin receptors. Both kinin formation and degradation take place locally in both extracellular tissue compartments, the interstitial space and the vasculature, as depicted in figure 5.

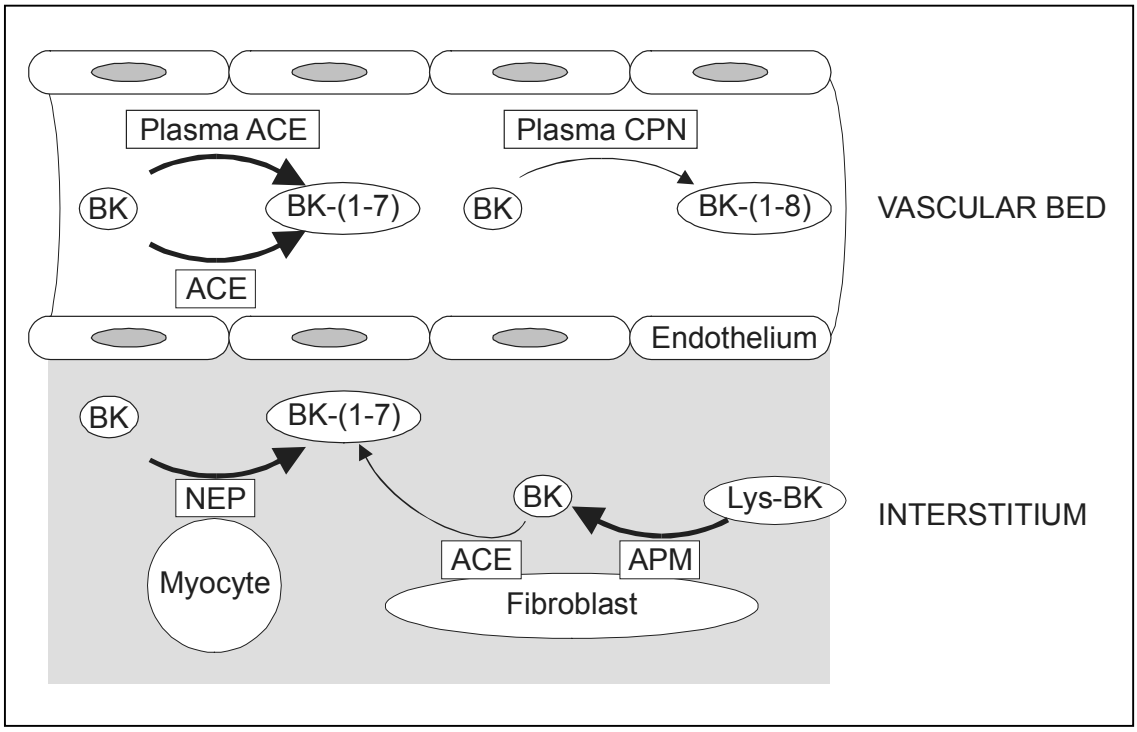

Figure 5. Schematic representation of the heart vascular bed and the interstitium.

1.1. Kinin metabolism in the circulation (II).

Figure 5 (II) summarizes the enzymatic degradation pathways of BK by human plasma. The major pathway consists of conversion of BK by ACE to BK-(1-7), an inactive metabolite. BK-(1-7) is further converted to BK-(1-5) by ACE, leading to accumulation of this active peptide. Less than 10% of BK is converted to the active metabolite BK-(1-8). The inhibition profile of this enzyme activity by MGEA is consistent with the enzyme responsible for this degradation being CPN-like activity (Skidgel *et al.*, 1988). In addition, our preliminary findings show that BK-(1-8) is also slowly degraded to BK-(1-5) by the endopeptidase activity of ACE (unpublished results).

1.1.1. Contributions of ACE and CPN to BK degradation in the circulation (II).

The results of earlier observations of the relative contributions of ACE and CPN to degradation of BK in plasma/serum were discrepant (Marceau *et al.*, 1981, Odya *et al.*, 1983, Sheikh and Kaplan *et al.*, 1989, Decarie *et al.*, 1996). The results presented in figure 4 (II) offer a plausible explanation for these findings. The K_m values of BK for CPN and ACE are 6-19 µmol/l and 0.2-1 µmol/l, respectively (Stewart *et al.*, 1981, Skidgel *et al.*, 1984 and 1988, Jaspard *et al.*, 1993) i.e. at BK concentrations below the K_m values of both enzymes, the hydrolysis of BK follows first-order kinetics. Since the physiological concentration of BK in plasma is well below the K_m values of the competing enzymes CPN and ACE, experiments performed with BK concentrations above the K_m values may lead to underestimation of the role of ACE in BK degradation in plasma (Marceau *et al.*, 1981, Sheikh and Kaplan *et al.*, 1989). Indeed, as shown in Figure 4 (II), with decreasing BK concentration, there was a gradual shift in the relative activity of the enzymes favoring ACE over CPN-like activity. At BK concentrations below the K_m values of the competing enzymes, the major BK-degrading enzyme was ACE.

1.1.2. Accumulation of BK-(1-5) in the circulation (II).

A novel finding in this study is that *in vitro* degradation of BK by plasma enzymes, mainly ACE, leads to accumulation of a pentapeptide, BK-(1-5). Until recently, this kinin was considered to be an inactive metabolite. However, in a recent report, BK-(1-5) has been found to be a selective inhibitor of thrombin-induced platelet activation (Hasan *et al.*, 1996). This finding suggests that BK-(1-5) may contribute to the constitutive anticoagulant nature of the intravascular compartment, and thus contribute to the cardioprotective nature of kinins independently of BK receptor-mediated effects.

1.1.3. The role of CPN in bradykinin metabolism in the circulation (II).

Our results imply that CPN (kininase I) is not the major BK-degrading enzyme in the circulation. CPN degrades BK to BK-(1-8), which was found to be very stable and was slowly degraded by endopeptidase activity of ACE. Therefore it may accumulate especially in a situation when the relative contribution to kinin degradation is increased. Its target receptor, BK-1R, is normally expressed in very low levels in the vascular tissues (Regoli *et al.*, 1981, Schremmer-Danninger *et al.*, 1998). However, BK-1R has been shown to be induced in pathological circumstances such as tissue trauma, inflammation, and anoxia (Nolly *et al.*, 1992, Proud *et al.*, 1994), and now, according to our results, in the SHR after the onset of hypertension and after the onset of HF (IV). Normally ACE is the key kininase in the circulation in addition to producing Ang II, therefore being an inhibitory target in both hypertension and HF. ACE inhibition makes CPN the major kininase in human plasma, most likely increasing BK-(1-8) plasma levels. This, with our findings of the increased amount of BK-1R in hypertension and HF, may mean that some of the cardioprotective effects of ACEi are exerted through BK-(1-8) and BK-1R.

1.2. The degradation of kinins in the heart tissue

1.2.1. Enzymatic degradation of kallidin in the heart tissue (I).

In this study we have shown that KD is rapidly degraded to BK by an aminopeptidase activity, the inhibitory profile of which closely resembled that of APM (Orawski *et al.*, 1989, Palmieri *et al.*, 1989). APM is widely distributed in tissues, being present, for example, on the plasma membrane of ECs and in SMCs (Palmieri *et al.*, 1989) and human respiratory epithelial cells (Proud *et al.*, 1994) but, to our knowledge, there are no previous reports of the activity of APM-like activity in human heart tissue. The role of APM in the regulation of local BK concentrations in tissues is currently unknown, but, owing to its high effectiveness and very low KD concentrations measured from tissues, (Duncan *et al.*, 2000) it does not seem to be a regulator of the production of BK.

1.2.2. The role of ACE in the bradykinin metabolism of the heart (I)

ACE is generally held to be the major BK-degrading enzyme and most of the work showing kinin-induced cardioprotection has been done using ACEis. In addition, *in vitro* experiments have demonstrated that purified ACE readily degraded BK to BK-(1-7) (Dorer *et al.*, 1974), and, in *in vivo* experiments, the use of ACEis increased the blood levels of BK in rats, suggesting that degradation of BK by ACE had been reduced (Campbell *et al.*, 1993). Therefore, our results, suggesting that ACE plays only a minor role in kinin metabolism in the membrane fraction of heart tissue, do not accord with the above concept of ACE being the major BK-degrading enzyme in the myocardium.

In vitro experiments may include pitfalls that lead to artificially low ACE activities in tissues. To rule out these possibilities, we performed several control experiments, the results of which are as follows. 1. The ACE activities in total heart homogenates were the same as in cardiac membranes, indicating that no ACE activity was lost during preparation of the cardiac membranes (data not shown). 2. The results presented in Table 4 (I) demonstrated that the cardiac ACE activities of patients receiving ACEis were not lower than those of patients not receiving ACEis or those of normal subjects. This finding, also shown by others (Urata *et al.*, 1990), most probably reflects the release of ACEis from ACE during the preparation of cardiac membranes. 3. Since endogenous ACEis, such as Ang-(1-7) and Ang-(1-9) may be present in heart tissue (Ikemoto *et al.*, 1989), we performed an experiment in which the activity of human plasma ACE was measured in the absence and presence of human cardiac membranes. We found that ACE activity was not affected by the presence of cardiac membranes (5 µg/assay), thus ruling out the presence of significant amounts of endogenous ACEis in the cardiac membrane preparations used in this study (data not shown). 4. The physiological concentrations of kinins in the heart tissue are below the K_m values of the competing enzymes NEP and ACE (Stewart *et al.*, 1981, Matsas *et al.*, 1984), and therefore we measured the degradation of BK by the cardiac membranes at a substrate concentration of 100 nmol/l which is well below the K_m values of both enzymes. The results show that at this concentration NEP was still the major BK-degrading enzyme, ACE playing a minor role (data not shown).

Our results clearly imply that the enzymatic activity of ACE in the human cardiac membranes is low compared to that of NEP. At least three possible explanations can be offered for the discrepancy between the earlier data of *in vivo* studies and the

present *in vitro* experiments. First, recent findings have demonstrated that ACEis directly potentiate BK receptor-mediated effects (Hecker *et al.*, 1997, Minshall *et al.*, 1997), making it possible that some of the observed effects of ACEis on BK metabolism are not due to inhibition of ACE. Second, most of the work on kinin induced cardioprotection has been done using different animal models and in contrast to our results in the human myocardium, it has been shown that, in the rat ACE is the main myocardial kininase (Dendorfer *et al.*, 1997, Kitakaze *et al.*, 1998). Third, localization of kininases in the myocardium may be very important. The only report directly addressing the localization of myocardial kininases has been performed in rats and states that ACE is located on the plasma membrane of the endothelium and in the interstitium, while NEP is located mainly in the interstitium (Dendorfer *et al.*, 1997). ACE is also responsible for the BK degradation in the plasma and it therefore seems that ACE is most likely the principal kininase in the vascular tree and that both plasma and endothelial ACE contribute to this.

1.2.3. The role of NEP in the bradykinin metabolism of the heart (I)

What, then, has been the previous experience regarding the roles of ACE and NEP in the degradation of BK in heart tissue? In studies with rat hearts, evidence has been found for both ACE- and non-ACE-mediated degradation of BK. However, in direct measurements, the concentration of BK-(1-7), a degradation product of both ACE and NEP, was not reduced by ACE inhibition although, in the same experiment, the concentration of Ang II was greatly reduced in the heart tissue (Campbell DJ *et al.*, 1994). It was later also shown that administration of NEPi to ACEi-treated rats did not modify blood or kidney BK peptide levels, as compared to ACEi alone. Importantly, ACEi did not affect BK peptide levels in the heart, while NEPi alone caused a 2-fold increase in cardiac BK levels and a 50% reduction in the cardiac BK-(1-7)/BK ratio in these rats (the ratio representing BK degradation by dipeptidases, such as ACE and NEP) (Campbell DJ *et al.*, 1998).

Our experiments with human cardiac membranes support the notion that the major BK-metabolizing enzyme in the heart interstitium is NEP. NEP is a metalloendopeptidase that is widely distributed in tissues. Its activity is highest in the epithelial cells of kidneys (Gafford *et al.*, 1983). NEP activity has also been found in the cardiovascular system; particularly on the plasma membranes of cultured rat CMCs (Piedimonte *et al.*, 1994).

In parallel with our report several others have appeared showing NEPi-induced cardioprotection through the kinin system. NEPi has been shown to induce classical reconditioning, and late PC (Schriefer *et al.*, 1996 and 2001), reduce infarct size, and protect against reperfusion arrhythmias resulting in increased survival (Yang XP *et al.*, 1997a, Rastegar *et al.*, 2000). Interestingly,when compared, an inhibitor of type A natriuretic peptide affected these effects very little, while HOE-140, a BK-2R antagonist, abolished most of them (Yang XP *et al.*, 1997a). In addition, NEP was shown to prevent isoproterenol-induced myocardial hypoperfusion and to improve myocardial oxygen consumption in the human heart by reducing BK degradation (Piedimonte *et al.*, 1994, Zhang X *et al.*, 1999a).
Importantly, NEPi has been shown to reduce the production of LVH after MI (Duncan *et al.*, 1999).

1.3. Clinical aspects (I, II)

Our data and the data obtained in the other laboratories, together, indicate that the kinin degradation is compartmentalized in the same way as kinin generation. In the vascular tree, ACE is the principal kininase, and CPN has a major impact only during ACE inhibition, and in the interstitium of the heart the key kininase is NEP, while ACE plays only a minor role. These data imply that, when aiming at increasing the kinin concentrations to exploit the kinin system for cardioprotection, a dual inhibition would give the best results. Indeed, in the recently published clinical trials IMPRESS and OVERTURE, which compared Omapatrilat (a combined inhibitor of ACE and NEP) with an ACEi in patients with HF, the combined inhibition was shown to be more effective than ACE inhibition alone in reducing mortality in these patients (significantly only in the IMPRESS) (Rouleau *et al.*, 2000, Packer *et al.*, 2002).

2. Bradykinin receptors in human and rat hearts (III, IV)

2.1. Myocardial expression of BK receptors in patients with end stage heart failure (III).

Since there were no differences in heart tissue kinin degradation between HF patients and their normal controls, we next moved to investigating the kinin receptors in our search for a possible direct role for the endogenous kinin system in HF pathogenesis. The present results, showing a differential expression of BK-2R in normal and failing hearts, are the first to suggest that BK-2Rs may be involved in the pathogenesis of human HF. In the light of studies with tKLK and BK-2R knockout mice (Emanueli *et al.*, 1999, Meneton *et al.*, 2001), the BK-2R signaling system seems essential for the regulation of myocardial growth. Thus, down-regulation of BK-2R expression in failing human hearts, as shown in this study, may reflect a maladaptive response in the pathogenesis of HF.

Interestingly, down-regulation of BK-2Rs in failing hearts is closely associated with a decrease in eNOS protein, one of the major downstream effector molecules in the BK/BK-2R-mediated cardioprotective signaling-pathway. Recently, a direct regulatory link between BK-2Rs and eNOS has been shown in normotensive rats (Bachetti *et al.*, 2001), suggesting that the level of eNOS and the amount of NO may depend on the level of BK-2Rs.

The observed down-regulation of BK-2R seems to affect not only the cells in the intramyocardial vascular tree, i.e. ECs and SMCs, but also the cells in the myocardial interstitium, i.e. the fibroblasts and CMCs. According to these results, the factor(s) responsible for the down-regulation do not seem to be cell-specific. However after further analysis of the data we concluded that BK-2R expression was strongest in the ECs of the human heart samples. Whether the receptor down-regulation is organ-specific is not known.

2.1.1. The effect of age on BK-2R expression (III).

Bradykinin has been shown to be cardioprotective against the development of LVH and subsequent HF (Wollert *et al.*, 1997, Emanueli *et al.*, 1999). Thus, a logical adaptive response to the onset of such a pathological process would be an increase in

BK-2R expression. Indeed, in normal hearts, we could show that the expression of BK-2R increases with age (data not shown), possibly as an adaptive response to an age-related physiological process of disease-independent fibrosis (Roffe *et al.*, 1998). These results support the presence of such an adaptive BK-2R response in normal hearts, and further suggest that the observed down-regulation of BK-2Rs in the failing hearts is not due to the greater age of this patient group. Thus, it is possible that the BK-2R expression is induced during the early stages of the disease (LVH), and that the observed receptor down-regulation occurs only later, at the stage of HF.

2.1.2. The effect of ACE inhibitors on the BK-2R expression (III).

Since ACEis are generally thought to increase the concentration of BK and all our patients in the HF group had received ACEi it is relevant to ask whether this ACE inhibition also may affect the level of BK-2R expression. Cell culture studies addressing the ligand-mediated feedback mechanism have been controversial (Faussner *et al.*, 1999, Bachvarov *et al.*, 2001). However it has been shown that BK levels in the human plasma (Cugno *et al.*, 2000) and human atrial tissue (Campbell *et al.*, 1999a) of HF patients treated with ACEis are similar to the BK levels of healthy persons. Furthermore, incubating human coronary artery ECs, which express BK-2R in a constitutive manner, with excess BK and ACEis for 18 hours did not affect their levels of BK-2R expression (Fig 1D, III). In addition the fact that the beneficial effects obtained by ACEis are inhibited by HOE-140, a specific BK-2R antagonist, does not support a suppressive role of ACEis for BK-2R expression. Lastly, in the present study, we analyzed a heart sample from an additional CHD patient who had not received any ACEis (see Figure 1 (III), black triangle), and did not observe any differences in BK-2R expression as compared with CHD patients treated with ACEis. However, although it appears unlikely that the observed down-regulation of BK-2R expression in the failing hearts is due to the presence of ACEis, we cannot completely exclude the possibility that chronic administration of ACEis may have a minor effect on the level of receptor expression.

It is not possible to collect human samples representing the important earlier stages in the pathogenesis of human HF. Therefore we next moved to using an animal model, namely SHR, to investigate the early expression of BK-2R.

2.2. Bradykinin receptors in the rat heart (IV)

2.2.1. BK-2R expression in rat models of acute and chronic pressure overload (IV)

The present results in SD rats show that during an acute pressure overload, induced by aortic banding and Ang II infusion, the expression of BK-2Rs was increased in the left ventricles. Similarly, in the SHR model, in which hypertension, LVH, and HF develop slowly as a consequence of chronic pressure overload, the increase in BK-2R expression correlated with the progression of compensated LVH without any significant fibrosis or LV dysfunction. These results suggest that BK-2Rs are involved in the cardioprotective regulatory processes of compensated LVH induced by both acute and chronic pressure overloads.

2.2.2. Down-regulation of BK-2Rs in the aging SHRs (IV)

Our present results also show that the mechanisms involved in the upregulation of the BK-2Rs, which is initially an adaptive response to pressure overload, may eventually fail. In the 20-month-old SHRs, a loss of BK-2Rs was associated with transition from compensated LV hypertrophy to HF, and was characterized by LV hypertrophy with an increased degree of fibrosis and diastolic dysfunction. Whether the down-regulation of BK-2R expression is a primary event or is secondary to the significant increase in fibrosis and the onset of diastolic dysfunction and HF cannot be deduced from the present experimental results.

The mechanisms involved in the down-regulation of the BK-2Rs in the failing SHRs remain to be established. The discrepant observation that the BK-2R mRNA level is highly induced in failing SHRs (at 20 mo), whereas the BK-2R protein level is reduced, suggests a presence of a post-transcriptional regulatory mechanism in these animals. Interestingly, BK-2R is not the only cardioprotective molecule having this kind of regulation, since a similar post-transcriptional regulatory mechanism has also been shown for the B-type natriuretic peptide (Suo *et al.*, 2002). Moreover, down-regulation of the BK-2R protein is not a general phenomenon, since other members of the G protein-coupled receptor superfamily, both deleterious and protective such as the AT-1R (Suo *et al.*, 2002) and type A natriuretic peptide (Suzuki *et al.*, 1993), are upregulated in the failing rat hearts.

Interestingly, similarly to the observed increase in BK-1R expression in the BK-2R knockout mice, (Duka *et al.*, 2001) loss of BK-2Rs in failing SHRs also leads to induced expression of BK-1Rs. Whether the increased expression of BK-1Rs in the failing SHRs is an attempt to compensate for the loss of BK-2Rs, is presently not known.

2.2.2.1. BK-2R expression in endothelial cells (IV)

BK exerts its cardioprotective effects through endothelial BK-2R and its downstream signaling cascades, leading to the production of NO, EDHF and prostacyclin (Hornig *et al.*, 1997). Keeping in mind the direct relationship between BK-2R and eNOS expression (Bachetti *et al.*, 2001, Cargnoni *et al.*, 2001), the present observations, showing down-regulation of BK-2Rs in the ECs, may indicate an important effect on the BK/BK-2R-mediated EC-derived cardioprotective potential, i.e. downregulation of expression and function of eNOS, thus potentially leading to endothelial dysfunction.

2.3. The cardioprotective potential of BK-2Rs (III, IV)

What then is the cardioprotective potential of BK-2Rs in the progression of HF? The kinin system has a cardioprotective contribution to most diseases leading to HF, such as hypertension and ischemic heart disease. In addition, it has direct effects on myocardial physiology and the pathogenesis of HF. BK counteracts endothelial dysfunction and promotes capillary growth, at the same time decreasing myocardial oxygen consumption. In addition, it promotes improvement in myocardial function by reducing the preload and improving cardiac metabolism and LV relaxation, resulting in increased contractile performance and cardiac output both at rest and in work.

In normal physiology, the kinin system, both the ligands and the receptor, seems to be upregulated whenever there is an event that affects the function of the heart adversely, as can be seen during PC and MI. We have here shown another example of this in the different modes of pressure overload, induced both pharmacologically and genetically. Thus, the initial increase in BK-2R expression observed after the onset of pressure overload may well represent an endogenous physiological cardioprotective system equivalent to the phenomena of PC and the enhancement of the kinin system in MI (Linz *et al.*, 1986, Vegh *et al.*, 1991). On the other hand, our data, showing a significant reduction in the number of BK-2Rs in both failing human and rat hearts, suggest that diseased hearts may not respond adequately to BK-mediated cardioprotection. Several studies with SHRs on ACEi treatment serve as an excellent example of this. In these studies, ACEi treatment affected the progression of fibrosis only if it was started before the onset of clinical HF, i.e. when the BK-2R expression was still upregulated (Pfeffer *et al.*, 1988, Boluyt *et al.*, 1995, Mitchell *et al.*, 1996).

Because kinins have the potential to affect both the pathologies leading to HF and the myocardial physiology during the pathogenesis of HF, it would be most appropriate to aim at somehow preserving or even reinforcing the kinin system. The only two mechanisms possibly targeted so far by medical therapies are the ACEi-induced resensitization of BK-2R and estrogen-induced BK-2R upregulation. In addition to these, BK-2R has been shown to be upregulated by other signals that are not directly applicable for clinical use, such as IL-1β. Therefore, in order to target the BK-2R with specific medical therapies, the exact mechanisms behind the regulation of BK-2R expression and the possible upregulatory signals need to be found. Only then it is possible to find use of their potential as cardioprotective effector molecules.

SUMMARY AND CONCLUSIONS

1. The present study shows that, in the human cardiac membranes, the most critical step in kinin metabolism, i.e. inactivation of BK, appears to be mediated by NEP. This observation suggests a role for NEP in the local control of BK concentration in heart tissue. Thus, inhibition of cardiac NEP activity could be cardioprotective by elevating the local concentration of BK in the heart.

2. The most critical step in plasma kinin metabolism, i.e. inactivation of BK, is mediated by ACE, while CPN plays a minor role. Thus, inhibition of plasma ACE activity offers cardioprotection by elevating the concentration of BK in the circulation.

3. Bradykinin type 2 receptors are decreased in the myocardium of end-stage HF patients. They were also shown to be increased by age in the cardiovascularly normal controls. The main effector molecule of BK, eNOS, decreased in the HF patients similarly to BK-2R. Our results are the first to suggest that BK receptors are involved in the progression of human HF.

4. The expression of cardioprotective BK-2Rs is increased during acute pressure-overload and compensated LV hypertrophy in rats. However, ongoing pressure-overload leads to a loss of BK-2Rs and transition to HF, with a dramatic increase in LV fibrosis and diastolic dysfunction. The results clearly support a role for BK-2Rs in the pathogenesis of HF.

ACKNOWLEDGEMENTS

First of all I want to warmly thank my instructors docent Ken Lindstedt and Jorma Kokkonen. Jorma taught me the basics of science and gave only clear lessons on many different aspects of science and medicine. Ken guided me through the latter part of the thesis, answered many questions I had on molecular biology and helped with everything in putting the thesis together. My warmest thanks are due to Naotaka Shiota, who taught me all the experimentation on molecular biology and with whom I spent many enjoyful moments discussing the essence of science and lots of other things during the last years. I am grateful to professor Petri Kovanen for sharing his experience on science and especially his positive attitude and sincere interest towards science. I also wish to express my thanks to Mikko Mäyränpää and Inka Liesmaa for their contribution on the thesis.

I am very grateful to professor Olli Vuolteenaho and docent Ilkka Pörsti for their thorough review of this thesis.

I wish to acknowledge the skillful technical assistance of Jaana Tuomikangas, Elina Kaperi and Suvi Mäkinen and expert secreterial work of Laura Fellman.I also express my thanks to all the rest of the Wihuri Research Institute personnel, with whom I enjoyed to work.

My thanks are due to professor Heikki Ruskoaho, Hanna Leskinen, Jaana Rysä, Marja Luodonpää and Risto Kaaja for their collaboration on the SHR studies.

I wish to acknowledge Markku Kupari, who knew where good science can be made.

I owe my sincere thanks to my parents Tapio and Kaisa Kuoppala, who were always ready and patient to answer my multitude of why's since I was two-years old. That, most likely, is the key behind my motivation to do science to begin with. I also want to thank my many friends, especially Matti Kuittinen, for keeping up the motivation and exchanging thought on science among other things.

My full-hearted gratitude belongs to my wife Sonja, who shared my vision on finishing the thesis and made enormous efforts especially the last year to give me the opportunity to write, and my children, Matias and Elli, for being patient with waiting for me the many moments I spent on making science and for making it all worthwhile.

Lastly and foremost I thank my Lord, Jesus Christ, for making me the way I am, for giving all the talents and the vision to make science, for teaching me how to finish things and lastly for guiding me through all the troubles on the way here and from hereon. *I can do all things through Christ, who strengthens me* (Philippians 4:13).

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